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FORMULATION OF PULLULAN-COATED LIPOSOMES AND RELEASE KINETICS OF WATER-SOLUBLE SOLUTES

BY

SANJAY SEHGAL

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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Dedicated to my parents,

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LIST OF ABBREVIATIONS

AraC	Cytarabine, Cytosine Arabinoside, Cytosine-β-D-arabinofuranoside
BP1-16	Peptide (23 aa residues, pI 7.1)
BSA	Bovine Serum Albumin
CH	Cholesterol
CHP	CholesterolPullulan
CE	Coating Efficiency
DCP	Dicetylphosphate
DDS	Drug Delivery System
DMPC	Dimyristoylphosphatidylcholine
DMF	Dimethylformamide
DOPC	Dioleylphosphatidylcholine
DPPC	Dipalmitoylphosphatidylcholine
DSC	Differential Scanning Calorimeter
EE	Encapsulation Efficiency
ESR	Electron Spin Resonance
FT	Freeze-Thaw
FATMLV	Freeze and Thawed Multilamellar Vesicle
GI	Gastro-Intestinal
HDL	High Density Lipoproteins
Ig	Immunoglobulin
μm	Micrometer
nm	Nanometer

NMR	Nuclear Magnetic Resonance
PDP	Potassium Dihydrogen Phosphate
PC	Phosphatidylcholine
pI	Isoelectric Point
psi	Pounds Per Square Inch
OPP	*O*-PalmitoylPullulan
R.T.	Room Temperature
RES	Reticulo Endothelial System
SC	Sodium Cholate
SG	Simulated Gastric Fluid USP
SI	Simulated Intestinal Fluid USP
SEM	Scanning Electron Microscopy
T_c or T_m	Phase Transition Temperature
TEM	Transmission Electron Microscopy
UV	Ultra Violet
V_{sed}	Sedimentation Volume

ABSTRACT

The formulation and characterization of uncoated and pullulan-coated liposomes containing cytarabine (AraC) or a small peptide ^{125}IBP1-16 (23 amino acid residues, pI 7.1) have been studied. Preliminary investigations led to selecting a liposome composition of dimyristoylphosphatidylcholine (DMPC), cholesterol (CH), and dicetylphosphate (DCP) at 3:1:2 mole ratio or dipalmitoylphosphatidylcholine (DPPC), CH, DCP at 3:1:0.5 mole ratio to encapsulate AraC, and dioleylphosphatidylcholine (DOPC), CH, and dimyristoylphosphatidylglycerol (DMPG) or DPPC, CH, DMPG at 3:1:0.25 mole ratio for ^{125}IBP1-16. The encapsulation efficiencies (EE) were 28 and 40 percent AraC in liposomes of DMPC:CH:DCP and DPPC:CH:DCP compositions, and 77 and 68 percent ^{125}IBP1-16 in DOPC:CH:DMPG and DPPC:CH:DMPG compositions, respectively.

Pullulan, which was derivatized with palmitoyl chains (OPP) or cholesterol chains (CHP), yielded maximum coating of liposomes from 0.5 percent solutions of OPP or CHP. Coating liposomes with OPP reduced the EE of AraC to 15 and 27 percent whereas CHP had no significant effect on EE. In case of ^{125}IBP1-16 however, adsorption of OPP or CHP at liposome surfaces did not alter the EE.

Coating liposomes with OPP or CHP improved their physical stability in sodium cholate solutions (1-16 mM) and significantly reduced the leakage of AraC. Likewise, reduced rates of release were observed in simulated gastric (SG, pH 1.2) and simulated intestinal (SI, pH 7.4) fluids USP at 37°C. The release profiles of AraC and ^{125}IBP1-16 in SG and SI fluids were characterized by an initial rapid release phase extending up to 2 hours (h) followed by first-order release kinetics extending to 12 h. Liposomes coated

with OPP decreased the amount of solute released during the initial phase and the first-order release rate constant (k_1) of AraC by 20 and 60 percent in SG fluids, and by 150 and 60 percent in SI fluids for the DMPC:CH:DCP and DPPC:CH:DCP liposomes, respectively. Similarly, CHP reduced k_1 by 100 percent in SG fluids for both liposome compositions, and by 30 and 70 percent in SI fluids for the DMPC and DPPC compositions, respectively.

Similarly, OPP decreased the fraction of ^{125}IBP1-16 released after 12 h by approximately 12 and 18 percent in SG or SI fluids, respectively, while CHP decreased it by 33 and 42 percent in SG fluids, and 28 and 33 percent in SI fluids for the DOPC:CH:DMPG and DPPC:CH:DMPG liposomes, respectively.

In terms of developing a liposome formulation that may be functional after oral administration, CHP-coated DPPC liposome compositions were better than the OPP-coated liposome compositions.

Chapter 1

INTRODUCTION

INTRODUCTION

The development and evolution of liposome research and its applications is a typical example of serendipity in scientific work. Liposomes were not discovered by researchers who were studying lecithin colloidal systems but by a researcher who used lecithin dispersions as a reagent in blood clotting studies. The unique properties of liposomes and their potential applications were fully recognized only in the mid 1960's by Alec Bangham and his colleagues, who realized that particles which were produced upon swelling of thin lipid films in water during agitation sequestered part of the solvent into their interiors and that the lipid bilayers represented models of biological membranes and permeability barriers for entrapped solutes. Since then, liposomes have been studied in both roles: as artificial biomembranes and as potential drug delivery systems. The work reported in this thesis describes the potential of liposomes as an oral drug delivery system.

A. Liposome Technology

1.1. The Liposome as a Drug Delivery System (DDS) - An Overview

Liposomes, discovered by *Bangham et al. (1965)*, form spontaneously when thin films of phospholipids are dispersed in aqueous media. Although used as models of biological membranes, their application as a DDS has gained considerable interest. There are many reasons for this depending on the specific application but, generally, it's their ability to entrap polar and nonpolar solutes, retain them for long periods of time, and increaase transport of some entrapped solutes across biological membranes that have given significance to these simple, biodegradable microparticles. More recent efforts have aimed at targeting liposomes to specific cells in the body by attaching receptor-specific ligands to the surfaces of liposomes. In the sections that follow, certain techniques that have been used to improve the functionality of liposomes will be reviewed.

Liposomes do not represent a panacea for the therapeutic administration of drugs, and the *magic bullet* approach is only begining to be developed. For the past two decades, the enthusiasm and high expectations of liposomes to accomplish what often has appeared as "miraculous feats" has subsided into the realism that these are physicochemical systems that must be better understood before they can be used medically in any meaningful manner. It was because they were easy to make and the components were readily available that the use of liposomes has been based largely on empirical results. Often, these were not reproducible or predictable in their *in vivo* behavior. It is now recognized that more fundamental research is required to fully characterize the properties of liposomes in terms of several criteria including particle size, composition, surface charge, membrane fluidity, interactions with and partitioning of a variety of molecular structures, and their chemical stabilities under different circumstances of preparation, storage and after administration. Often overlooked is the susceptibility of some phospholipids to oxidation or hydrolysis to form degradation products which are potentially toxic or which disrupt the organized structures of the liposomes. Evidence of the wide range of applications of liposomes is manifest in the growing list of new terms to describe the structure or intended application of liposomes. Thus, the following terms have appeared in the literature: crytosomes, dermasomes, immunoliposomes, niosomes, ninjasomes, proteoliposomes, pharmacosomes, and stealth liposomes. Liposomes have generally been administered by parenteral routes including intravenous, intramuscular, subcutaneous, intraperitoneal injections and more recently also by the intratracheal route. The oral route of administration has not yet been perfected. It is suggested, however, that should this occur, they be referred to as *orasomes*.

It would appear that one of the biggest challenges in liposome drug delivery is to develop a functionalized liposome for oral administration. Studies so far have not been encouraging but these have not taken into consideration any of the basic requirements of a liposome to be successful following oral administration. These include liposome stability, bioadhesiveness to the epithelium at specific regions of the GI tract, and the propensity of being endocytosed at the site of absorption or, at least, to slowly release the depot of drug at the site of absorption. Some promising formulation approaches to achieve these objectives are the subject of this thesis.

1.2. Composition of Liposomes

Phospholipids are the major structural components of biological membranes in addition to sphingolipids, glycolipids, and steroids. Hence, the most common phospholipids used in preparing liposomes are the phosphatidylcholines (PC) or *lecithins*. These are amphipathic molecules consisting of a glyceryl-*sn*-phosphocholine esterified by two acyl chains at positions R_1 and R_2 of the glycerol backbone. The acyl chains may be the same (symmetric) or different (asymmetric) yielding PCs with different physical properties including gel and liquid-crystalline phase transition temperatutes (T_m). Other phospholipids vary by the nature of the polar head group attached to the phosphatidyl moiety and by the degree of saturation/unsaturation of the hydrocarbon chains (Figure 1.1).

Molecules of PC carry an overall neutral charge over a wide range of pH because of their zwitterionic character. They have very poor water solubilities and above their solubility coefficients, they align themselves tail-to-tail in planar bilayer sheets in order to minimize unfavourable exposure of hydrocarbon chains to the aqueous environment. The bilayers orient themselves in a closed, spherical arrangement. Many such bilayers may be present separated by aqueous compartments within a liposome structure, *i.e.*, multilamellar liposomes.

Liposomes, like biological membranes, can contain mixtures of phospholipids, as well as, include lipids and proteins which give them unique properties (Figure 1.2, from Liposomes in Biological Systems, G. Gregoriaids and A. C. Allison, eds.). They have been prepared from neutral phospholipids as PC, sphingomyelin (SM) and phosphatidylethanolamine (PE). SM liposomes are more condensed and stable than PC liposomes. Sterols, charged adducts and mixtures of different phospholipids are often included in liposomes to give them properties such as improved stability, encapsulation efficiencies of solutes, and to alter their *in vivo* disposition. Negatively-charged liposomes can be prepared with phosphatidylserine (PS), phosphatidic acid (PA), phosphatidyl glycerol (PG) and dicetylphosphate (DCP) whereas positively-charged liposomes have been prepared with stearylamine (SA) and more recently with less toxic cationic agents suchn as translectin, DOTAP and DOTMA. Sterols, in particular cholesterol (CH), being amphipathic inserts into the membrane with its 3-β-hydroxyl group oriented towards the aqueous surface and the aliphatic moiety inserted within the acyl chains of the bilayer (Figure 1.2, from Liposomes in Biological Systems, G. Gregoriaids and A. C. Allison).

1.3. Liposome Preparation Methods

Liposomes range in diameter from 20 nm to as much as 10 μm. The aqueous compartment of liposomes may be bound by a single bilayer membrane (unilamellar) or may be composed of many concentric bilayers separated by aqueous compartments (multilamellar vesicles). Typically, the liposomes are classified based on their size into following types:

- *Small unilamellar vesicles (SUVs)*, diameter range 20 nm to 50 nm,
- *Large unilamellar vesicles (LUVs)*, diameter range 50 nm to 1000 nm,
- *Multilamellar vesicles (MLVs)*, diameter range 200 nm to approx. 10 μm and,
- *Multivesicular vesicles (MVVs)*, diameter range 1 μm to approx. 10 μm.

1.3.1. Hydration Method

Several methods of liposome preparation have been reported over the years. The method that was initially reported was the hydration method (*Bangham et al., 1965)* that is still widely used today. It involves the hydration of a thin dried film of the chosen lipid mixture deposited on a glass surface, such as the walls of a round-bottom flask or on glass beads placed in the flask in order to increase the surface area. A solution of lipids in a suitable organic solvent (*e.g.* chloroform) is evaporated to dryness with the aid of a rotary-evaporator. The last traces of solvent are removed by leaving the flask in a vacuum oven at approximately 45°C for several hours. Subsequently, a prewarmed aqueous solution of the solutes to be entrapped is added and vortex-mixed at an elevated temperature at least 10°C above the T_c (phase transition temperature) of the constituent phospholipids. As the lipid films hydrate, they swell and peel off from the glass surface in sheets forming myelinic structures and MLVs. After a brief annealing period, the liposome mixture is hand shaken or vortex-mixed, and then extruded through polycarbonate filters or freeze-thawed to improve size homogeneity.

Chloroform is the most commonly used organic solvent used to solubilize lipids. However, when charged lipids are included, solvent blends such as chloroform:methanol (70:30 v/v) may facilitate the solubilization process. The hydration of dried film normally occurs more rapidly at a temperature above the T_c of the major phospholipid and the rate of hydration is a function of the lipid composition. The rate of hydration can be improved by including charged lipids such as DCP, PA, PG or SA in the bilayers.

1.3.2. Detergent Removal Method

This method is considered to be a very mild treatment suitable for encapsulation of proteins which may become denatured or inactivated by organic solvents and subsequent particle size reduction treatments. A detergent micellar solution and a dispersion of MLVs are equilibrated until mixed micelles are formed. The detergents that have been routinely used for this purpose include sodium cholate (SC), octylglucoside and alkylacyloxypolyethylenes (*e.g.* Triton X-100) (*Kagawa and Racker, 1971*). The solution is then dialyzed (*Mimms et al., 1981*) or treated by gel filtration (*Hauser et al., 1976*) to remove the detergent molecules. Under appropriate conditions, small MLVs or SUVs are produced in which the proteins/solutes can be entrapped. The process causes about a six-fold dilution of the liposomes but they may be subsequently concentrated by filtration or freeze-drying.

1.3.3. Injection Methods

Injection of either an ethanolic solution or an etheral solution of lipids into an aqueous phase at different temperatures of the organic/aqueous phases results in liposome formation. For example, *Batzri and Korn (1973)* reported that the injection of 5 mM egg PC dissolved in ethanol into a well-stirred aqueous phase produced SUVs with diameters of approximately 30 nm. Subsequently, the ethanol was removed by dialysis.

Alternatively, *Deamer and Bangham (1976)* described the slow injection of an ether solution of lipids into a warm aqueous phase which produced LUVs having a high encapsulation efficiency from which the ether was removed by evaporation. Any residual volume of ether could be removed by dialysis or gel filtration.

1.3.4. Reverse-Phase Evaporation Vesicles (REVs)

A variation of the ether injection method uses an organic solvent of lower volatility which is emulsified with the aqueous phase to form a water-in-oil (w/o) emulsion or a w/o/w multiple emulsion. *Szoka and Papahadjopoulos (1978)* described a reverse phase evaporation method which also yielded LUVs. The term REV was used because the process involved the formation of a w/o emulsion which was the reverse of the standard oil-in-water emulsion. The lipids are dissolved in a volatile organic solvent (*e.g.*, diethyl ether, diisopropyl ether and/or chloroform). The aqueous phase is added to the organic phase, and the mixture is sonicated to form an w/o emulsion. The organic phase is then slowly removed by rotary evaporation under reduced pressure to form a gel. This is then diluted with an aqueous medium to form LUVs.

1.3.5. Criteria for Selection

The desired encapsulation efficiency, liposome size, number of lamellae, and nature of the solute determine the method to be selected for liposome preparation. It is important when the solute is a peptide or a protein drug to avoid a technique which exposes it to organic solvents, extremes of temperature, and prolonged exposure to probe sonication. MLVs prepared by hydration are the most convenient to prepare and, although the size distribution can be large, reproducibility in behaviour of the liposomes is high as long as attention is paid to the temperature of hydration, time period for votex-mixing, and the time in which the liposomes are exposed to bath sonication. The encapsulation of hydrophobic drugs is generally greater than hydrophilic drugs in MLVs because of the high lipid:water ratio. For hydrophilic drugs, however, higher EE are often obtained in unilamellar vesicles because of the higher water:lipid ratios.

1.4. Properties/Characteristics of Liposomes

1.4.1. Size Determination

Liposome size and size distribution affects many of their properties such as aggregation, stability, absorption, biodistribution and *in vivo* clearance rate. The size of liposomes to be used depends to a large extent upon the intended application, but takes into consideration the route of administration and the encapsulation efficiency required. Sonication of MLVs to produce SUVs has been widely practised to reduce particle size for intravenous administration. Probe sonication is more efficient than bath sonication but introduces contaminants into the liposome sample such as titanium particles from the tip of the probe. The temperature must be controlled to avoid deterioration of the liposome components. Another increasingly popular method of reducing particle size and increasing size homogeneity is by filtration through a single or a series of polycarbonate membranes of decreasing porosities, also referred to as extrusion. The Extruder® (Lipex Biomembranes, Vancouver, BC) is a commercial instrument used for this purpose. Alternatively, reduction of liposome size can also be accomplished using the homogenizer principle. Among the earliest of these is the French Press which forces a liposome dispersion through a small orifice at pressures as high as 25,000 psi. However, the elevated temperatures and the lack of reproducibility of particle size distributions are major drawbacks. More recent commercial instruments of this type are the Microfluidizer® for industrial scale quantities of liposome and Lipsofast® or Emulsiflex® for small research/clinical based sample sizes.

Many techniques have been used to characterize the mean particle diameter and the size distribution of liposomes. Light microscopy is convenient for examining the size distribution of large vesicles (above 0.5 μm) and even particle shapes but it is unsuitable for resolving the size of smaller vesicles (*Nassander et al., 1990*). Two other techniques have been used for small liposomes, one based on quasi-elastic laser light scattering (*Ruf et al., 1989*) and another involving direct observation of the liposomes under an electron microscope following negative staining.

1.4.2. Encapsulation Efficiency of Solutes

The amount of a lipophilic solute encapsulated in liposomes is generally a function of the concentration of the lipid and of the lipid-water partition coefficient. Polar or hydrophilic solutes are chiefly encapsulated within the aqueous compartments of the liposomes, hence their encapsulation efficiency (EE) is a function of the concentration of the drug in the aqueous phase and the sequestered aqueous volume which may be influenced by the addition of charged adducts in MLVs. Liposomes (MLVs) possessing negatively or positively-charged bilayer surfaces have larger aqueous compartments than their neutral counterparts due to the electrostatic repulsion of the bilayers, and hence, can achieve higher EEs. Also, solute molecules carrying an opposite charge than the bilayers will experience electrostatic binding, and hence, contribute to the total amount of solute molecules encapsulated. A method to obtain EEs approaching 100 percent utilizes a pH-gradient loading technique (*Cullis et al., 1989; Madden et al., 1990)*. It involves equilibrating a solution of the cationic drug at a higher pH (*e.g.*, 8.0) with liposomes containing an aqueous medium at lower pH (*e.g.*, 4.0). The solute is transported from an area of lower aqueous solubility to an area of higher solubility (due to a greater fraction of ionized solute, pH-partition theory), causing the solute to concentrate inside the liposome bilayers. This method of loading solutes into liposomes has been found to be useful for doxorubicin and some of the local anesthetics.

The process of freeze-thaw (FT) cycling can lead to increased entrapment efficiency of hydrophilic solutes. The EE of small non-electrolyte solutes improved from 5 to 65 percent following five freeze-thaw cycles (*Mayer et al., 1985*). This process results in rupture and fusion of the vesicles and a reduction in the number of the lamellae during which time the solute equilibrates between the inside and outside bilayers resulting in improved EE.

Similarly, *Kirby and Gregoriadis (1984)* have reported that as much as 40-50 percent of small molecular weight solutes, such as glucose, could be entrapped when the preformed liposomes are lyophilized, and then rehydrated to obtain dehydrated-rehydrated vesicles (DRVs). This process may be repeated several times to maximize the EE. This method is in principle similar to the FT method (*Gregoriadis et al., 1990*). The dry lyophilized material is normally rehydrated with a much smaller volume of water than that from which it was dried. As mentioned previously, care must be taken that the liposome treatment methods to increase EE of protein/peptide drugs must not destroy their intrinsic activities.

1.4.3. Stability of Liposomes

Stability usually refers to storage stability and stability in different environments (*e.g.*, GI tract, plasma, skin). One measure of liposome stability has been the evaluation of rate of leakage of entrapped solute, *e.g.*, sodium carboxyfluorescein. In addition, the turbidity changes at either 400 nm (*Regen et al., 1980*) or 550 nm (*Defrise-Quertain et al., 1985*) in response to increasing concentrations of detergent are also an indication of the degree of liposome stability.

Membrane solubilization by detergents is due to the formation of micelles at concentrations above their critical micelle concentration. When such membrane-solubilizing detergents are added in increasing amounts to the phospholipid membranes, more and more detergent will be incorporated into the bilayers forming mixed-micelles (micelles containing lipid components in addition to the detergent). The susceptibility to disruption appears to be greatest when the phospholipids are in the liquid-crystalline state. The inclusion of CH generally improves the stability of liposomes.

The liposomes represent a heterogenous system with potential chemical and physical stability problems (*Frokjaer et al., 1984*). Often ignored is the chemical stability of liposomes *in vitro* that pertains to their structural integrity. At elevated temperatures and extremes of pH (a minimum hydrolysis rate is found at pH 6.5), phospholipids are labile to hydrolysis at the C2-position of the glycerol moiety resulting in the formation of lysophospholipids and free fatty acids that greatly increase the permeability of liposomes (*Grit et al., 1989*). Unsaturated phospholipids, including egg yolk or soya bean-derived PCs, with unsaturated fatty acids are susceptible to oxidation during liposome preparation, particularly if sonication is used, if they are not stabilized by antioxidants such as α-tocopherol or ascorbic acid derivatives. The saturated chain phospholipids, however, are much less prone to oxdation.

Delivery of drugs *in vivo via* liposomes is obviously dependent on retention of their physical integrity until they reach their target sites or serve as a depot of drugs. Following systemic administration, the liposome stability pertains to the resistance offered by the vesicles against disruption by serum proteins, particularly the HDLs, and by enzymes and hormones. In the GI tract, however, they must not be readily disrupted by the changing pH (1-8) and by enzymes such as pepsin, trypsin, chymotrypsin, amino and carboxy-peptidases and the bile salts that have the ability to form mixed micelles with liposomes. The stability of liposomes made with the phospholipids, DMPC, DLPC and DPPC against phospholipase-A_2 was studied by *op den Kamp et al., (1974)*. They showed that the enzyme hydrolysed the membranes only at temperatures close to the T_c of the constituent phospholipids where the lipid bilayers are highly disorganized. However, at temperatures both above and below the T_c, the enzyme had little effect on the integrity of liposomes (*op den Kamp et al., 1974; Richards and Gardner, 1978*).

1.4.4. Phase-Transition Temperature

As the PC bilayers are heated, the hydrocarbon chains undergo a transition from an ordered solid or gel state ($<T_c$) to a fluid or liquid-crystalline state ($>T_c$) with disordered hydrocarbon chains where the freedom of movement of individual molecules is higher (Figure 1.3).

The T_c of phospholipids is dependent on the *Van der Waals* forces of attraction between the hydrocarbon moieties, interactions between the polar head groups, extent of hydration, and hence, varies between -20 and 60°C for different commonly used phospholipids. In general, increasing the fatty acid chain length or increasing the saturation of the chains increases the T_c, while introducing *cis* double bonds or branched chains tends to lower the T_c.

The addition of CH to PC membranes alters the position of the main T_c peak along with peak bradening. For instance, the T_c of DPPC increases from 41°C to 44°C after the incorporation of 33 mole percent CH. With increasing concentration of CH, the T_c disappears at 50 mole percent CH (*i.e.*, 1:1 ratio of DPPC:CH) (*McMullen et al., 1993*). Thus, CH alters the fluidity of the phospholipid membrane both below and above the T_c in such a manner that below the T_c, the fluidity of the bilayers is increased presumably due to decreased packing of the phospholipid molecules while above the T_c, the fluidity of the bilayers is decreased, presumably due to stronger *Van der Waals* forces of attraction (*Demel and DeKruyff, 1976; McMullen et al., 1993*). These changes in fluidity due to CH addition are paralleled by changes in membrane permeability which decreases by high cholesterol:PC ratios at temperatures higher than T_c but increases at lower temperatures.

1.4.5. Bilayer Rigidity/Permeability

Bilayer rigidity influences the biodistribution and biodegradation of liposomes. The bilayer permeability to entrapped hydrophilic low molecular weight solutes depends primarily on the size and charge of the drug entrapped, physical state of the membrane including bilayer rigidity, and environmental factors such as temperature, pH and ionic strength of the medium. Characterization of liposome formulations by DSC, fluoresence depolarization, NMR and ESR can provide valuable information regarding the magnitude of interaction of the solute with lipid bilayers. An indication of the bilayer rigidity can also be obtained by using fluorescence techniques. *Crommelin and Van Bommel (1984)* observed that, carboxyfluorescein, a hydrophilic marker leaked out much faster from fluid-state than from gel-state bilayers and that the leakage rates were highest at the T_c.

Bilayer rigidity is one of the major features which has been found to correlate with the leakage rates of entrapped solutes *(Crommelin and van Bommel 1984)*. Generally, liposome bilayers are more permeable to small water-soluble solutes (*e.g.*, glucose, cytarabine) than to large molecules (*e.g.*, macromolecules).

1.4.6. *In Vivo* Disposition of Liposomes

Liposomes administered *in vivo* are subject to a number of interactions which can determine their rate of clearance and the degree of organ uptake. Under normal circumstances, when lecithin/cholesterol liposomes are injected into the bloodstream, they are taken up to a large extent by organs rich in cells of the RES system (*e.g.* liver, spleen, lungs, blood and bone marrow). When liposomes are injected into the bloodstream, their surfaces rapidly become coated with plasma proteins including apolipoproteins, α, β and γ globulins, IgG, fibronectin, clotting factors and albumin, all of which may affect the way in which the liposomes are recognized by the phagocytic cells and, hence, the rate at which they are cleared from the bloodstream by the RES (*Juliano and Lin, 1980*).

In the late 1980's, a major advance was achieved when it was realised that inclusion of certain lipids in the bilayer can effectively prolong blood circulation times of liposomes (*Allen and Chonn, 1987*). The first approaches were empirical, based on mimicking the outer composition of red blood cells that contain shielded sialic acid moieties on their surfaces and, hence, are not recognized as foreign by the mononuclear phagocyte system (MPS). The next step was achieved by inclusion of phospholipids with a synthetic hydrophilic polymer headgroup. This work concentrated, in analogy with earlier successes with proteins (*Abuchowski et al., 1977; Nucci et al., 1991*) on polyethyleneglycol chains. The reduced MPS uptake and prolonged blood circulation are achieved by steric stabilization: reduction of particle interactions leading to aggregation and fusion. In addition to this approach of preventing recognition of liposomes by the MPS, other alternative approaches have been considered to modify their surface characteristics.

Following oral administration, some data indicate that endocytosis is more likely to occur for gel state liposomes (*Papahadjopoulos et al., 1975*) in the ileum, endocytosis of both fluid and gel-state MLVs and SUVs has been reported (*Pagano and Weinstein, 1978*). This mechanism of uptake has also been reported to increase the incorporation of liposomes in everted rat intestinal sac studies (*Rowland and Woodley, 1981*), which has generated further interest in developing liposomes for oral administration. Most recent attempts have been at targeting liposomes to the Peyer's patches in order to improve their uptake from the GI tract. Also, certain proteins as tomato lectins have been attached to the surface of liposomes to target them to specific sites in the GI tract.

1.4.7. Site-Specific Liposomes

The liposome composition can be devised so that the release of entrapped solutes is site-specific, in response to the environmental conditions, either at the gross anatomical level, at the cellular or at the sub-cellular levels. Hence, for site-specific delivery, temperature-sensitive, pH-sensitive and target-sensitive liposomes have been developed.

The **temperature-sensitive liposomes** take advantage of the fact that liposomes leak their aqueous space contents readily at the T_c of the membrane lipids. *In vitro* or *in vivo*, this leakage is augmented by the presence of certain plasma proteins as high density lipoproteins (HDLs), that bind to the membrane and help to destabilize it resulting in a release of the contents at the T_c. Normally, such liposomes consisting of a mixture of DPPC and DPPG are designed to be stable at 37°C, but will break down as they pass through an area of the body in which the temperature is raised to 40°C, *e.g.* tumour sites, inflammed area or areas subjected to local external heating. They can release their contents while flowing through a heated organ or upon their localization in the target organs.

The **pH-sensitive liposomes** are similar in approach to that described in the target-sensitive liposomes. Using PE as the major phospholipid, the membrane is stabilized by the addition of materials which are charged and hence stable at neutral pH ($\simeq 7.0$), but lose their charge at low pH (≤ 5.4) along with their ability to stabilize the membrane. Such compounds include fatty acids, palmitoylhomocysteine, cholesterol hemisuccinate and N-succinyl PE. Judicious mixing of these materials with PE in a proportion of 10-30 mole percent can result in liposomes which remain intact at pH 7.0 but will fuse when taken down to pH 5.4 (*Wang and Huang, 1987*).

The underlying principle of **target-sensitive liposomes** is that they are composed of lipids which form a membrane which is intrinsically unstable (*e.g.* unsaturated PE, which naturally adopts the hexagonal-inverted micelle structure in preference to bilayer sheets). This membrane is stabilized by the presence of proteins anchored in the membrane by covalent attachment to fatty acid chains or other lipid molecules. Typically the protein employed is a specific antibody; if the antibody-bearing vesicles meet their target cells, the antibodies will bind to multiple epitopes on the antigen presenting cell(s) and in the process, the antibody molecules will be drawn together and form a cap, leaving a large expanse of free membrane exposed and highly susceptible to destabilization. Release of the entrapped materials can take place in the vicinity of target cells and hence becomes available to them without the need for ingestion of the liposomes.

Such an approach is particularly appropriate for delivering materials which exert their effects on cells by binding to cell surface receptors (γ-interferon) or for delivery to cells whose endocytic activity is reduced, *e.g.*, in response to viral infections (*Ho et al., 1986*).

1.5. Successful/Promising Applications of Liposomes as a DDS

There are numerous examples of the successful application of liposomes to improve drug stability, reduce toxicity, increase their absorption and targeting to specific tissues and/or organs. Only recently has the number of liposomal products reaching Phase III clinical trials increased significantly. One of the first successes has been their passive targeting to phagocytic cells in order to treat diseases of the RES such as leishmaniasis (*Croft, 1986*) and to treat systemic fungal infections (*Sculier et al., 1988*). Liposome encapsulation of meglumine-antimoniate resulted in more than a 700-fold increase in its therapeutic efficacy against leishmaniasis compared to the free drug (*Alving et al., 1978*). Table 1.1 reviews some of these disease-types wherein liposomal encapsulation has shown promising applications (taken from *Lasic, 1993*). Some other successful applications of liposomes as pharmaceutical products include:

- Kerasal® (liposomal dispersion) for the treatment of keratinitis, Lipopharm, Inc., Montreal, Canada;

- Limethason*, (Lipo-dexamethasone), an anti-inflammatory agent, Green Cross Co., Japan;
- Pevaryl*, 0.2 and 0.5 % lipogels, topical anti-mycotic liposome gels containing the antifungal agent econazole, Cilag A.G., CH-Schaffhausen, Switzerland.

An important challenge has been to decrease the Amphotericin-B associated nephrotoxicity by a liposomal formulation (AmBisome, Vestar, Inc., D-Wolfenbüttel, Germany). This product has shown a several-fold decreased nephrotoxicity and improved efficacy in the treatment of systemic fungal infections (*Lopez-Berestein, 1988 & 1989*). AmBisome consists of SUV vesicles in the size range 45 to 80 nm that are composed of PC, CH, distearoylglycerol, and Amphotericin B (molar ratio 2:1:0.8:0.4), with a lipid/drug ratio of 9.5. Several hundred patients have already been treated in Europe and it has shown good tolerance and efficacy. It is now going through phase III clinical trials in the U.S.A. In addition to its liposomal product, Amphotericin B has also been formulated as micellar and as a colloidal dispersion (Amphotericin B colloidal dispersion, ABCD*, Liposome Technology Inc., CA). This formulation has been commercially available on a name-patient basis in Europe since 1992 under the commercial name Amphocil (*Lasic, 1993*).

Other systemically-active liposome products that are currently in clinical trials include doxorubicin (Doxil, Liposome Technology Inc., phase III clinical trials in the U.S.A), methotrexate (Mytek$_{G65}$, The Liposome Company, NJ, USA), cis-platinum, mitoxantrone, calcein, parathyroid hormone, and muramyl-tripeptide. Other solutes that are at the pharmaceutical development stage include clindamycin (Lipopharm, Inc., Montreal), clobetazone and epirubicin (The Liposome Company, NJ). Recently it was reported that liposomal daunorubicin has been sucessfully used in the treatment of patients with HIV-associated Kaposi's sarcoma (*Presant et al., 1993*)

B. Lipid and Surface-Modified Liposomes

1.6. Polymerized Liposomes

Polymerized liposomes differ substantially from conventional liposomes in their stabilities towards mechanical and chemical stresses. They can retain their physical integrity even after mild ultrasonication and exposure to organic solvents and detergents, *e.g.*, the bilayer structure remained intact in 50 percent ethanol, and the leakage rate of entrapped solutes were greatly reduced (*Hupfer et al., 1983; Juliano et al., 1984*). *Okada et al. (1993)* have also demonstrated similar effect from 1,2-di(octadecadienoyl)-3-PC liposomes *in vitro*. Such polymerized liposomes also have a greater resistance to osmotic pressure changes. However, open questions remain, such as the degree of biodegradability and the degree to which the release of the drug can be controlled *in vivo* (*Krause et al., 1987*).

Polymerized vesicles are prepared with any of the available conventional liposome preparation techniques by using polymerizable lipids having a wide range of sizes and lamellar states. Polymerization is catalyzed by ultraviolet (uv) light or some free radical initiators. UV catalysis is the simplest and most widely used method, *e.g.* lipids containing conjugated diacetylene, conjugated diene, methacrylate and thiol groups can be polymerized by this method (*Day and Ringsdorf, 1978*). MLVs and LUVs containing those compounds polymerize below the T_c of lipids upon brief exposure to irradiation. SUVs have a high surface curvature because of their small sizes, and hence, show substantially reduced photopolymerizability (*Lopez et al., 1982*). Alternatively, condensation polymerization with the aid of standard free radical initiators, such as azobisisobutyronitrile (*Ohno et al., 1987*) or hydrogen peroxide (*Regen et al., 1983, Samuel et al., 1985*) have yielded polymerized liposomes.

Polymeric liposomes have also been prepared from pre-polymerized lipids by polycondensation (*Kunitake et al., 1981 & 1984; Elbert et al., 1985; Neumann and Ringsdorf, 1986*). An advantage of this method is that impurities are not as likely to be introduced since the polymerization occurs before liposome assembly. The polymerized lipid can be purified beforehand by conventional solubilization-precipitation procedures. The main disadvantage is that only relatively low molecular weight polymers are able to form liposomes, which is unlikely to result in improved liposome stability.

1.7. Polymer-Coated Liposomes

Many approaches based on surface modification have been considered to decrease interaction with biological components and thereby increase the blood circulation. Some of the earliest attempts explored methods of coating liposomes with natural products such as proteins, polysaccharides (*Sunamoto and Iwamoto, 1986; Sato et al., 1988*) and glycoproteins (*Torchilin et al., 1980*).

Coating liposomes with polysaccharides has been another approach used to stabilize liposomes. Liposomes coated with polysaccharides, such as *O*-palmitoylpullulan (OPP) or cholesterolpullulan (CHP) have shown greater stability than conventional liposomes (*Sunamoto et al., 1983; Sato and Sunamoto, 1992*). Also, carboxymethylchitin-coated liposomes were shown to offer greater resistance to solubilization by detergents than the uncoated liposomes (*Kato and Kondo, 1987; Dong* *and Rogers, 1991*). Recently, *Ringsdorf et al. (1993)* reported that coating liposomes with polymers such as poly-(N-isopropylacrylamide) not only strengthened the bilayer, but also triggered controlled diffusion of entrapped solutes. There are essentially four types of polymer-coated liposomes based on the mechanism of interaction between the polymer chains and the lipid bilayers (Figure 1.4).

1. Adsorption of the polymer molecules at the liposome surfaces by hydrophilic or ionic forces (*Seki and Tirrell, 1984*);
2. Fixation of polymer molecules by hydrophobic anchor groups which penetrate lipid bilayers (*Martin and Papahadjopoulos, 1982; Sunamoto et al., 1987 and Elferink et al., 1992*);
3. Fixation of polymerizable, water-soluble molecules to the liposome surfaces *via* salt formation followed by spontaneous polymerization (*Aliev et al., 1984; Fukuda et al., 1986; Dong and Rogers, 1992*).
4. Adsorption of polymerizable, water-soluble molecules on liposome surfaces following cleavage of polymer chains.

Liposomes coated with polymers via *hydrophilic adsorption* result from incubation of the polymer solution with preformed liposomes. A modification of the REV method has been used to deposit the polymer at the surfaces of liposomes during their formation. In this manner, non-specific interaction between polymer and liposome is increased. Carboxymethyl chitin has been successfully used to coat liposomes by this technique (*Kato et al., 1984; Izawa et al., 1986; Kato and Kondo, 1987; Dong and Rogers, 1991*).

In order to increase the polymer-liposome surface interactions, polysaccharides or poly(carboxylic acid)s have been derivatized with *hydrocarbon anchors* that hold the polymer onto the liposome surfaces. Naturally-occuring polysaccharides that have been derivatized with palmitoyl or cholesterol groups as hydrophobic anchors include pullulan, dextran, amylopectin, and mannan (*Takada et al., 1984, Arnold et al., 1986; Sunamoto et al., 1987 & 1988; Sato, 1990*). This improves the retention of the polysaccharide at the liposome surfaces, particularly when the liposomes are being ultracentrifuged or filtered by gel permeation chromatography. The chemical synthesis of both of the polysaccharide derivatives used, OPP and CHP, following the procedures of Hammerling and Westphal (1967) and Sato (1990) are shown in Figures 1.5 and 1.6, respectively.

It has been shown that liposomes can be coated by polymers *via* a *salt bridge* and autopolymerization at the liposome surface (*Ringsdorf and Schlarb, 1988*). Thus, salt formation between cationic 4-vinylpyridine and negatively-charged liposomes followed by autopolymerization of the 4-vinylpyridine under normal light conditions produced poly(4-vinylpyridine)-coated liposomes (*Dong and Rogers, 1992*). Such a liposome system had greater stability in isopropanol and sodium cholate solutions. Such liposomes also manifested reduced leakage rate of acetyl salicylic acid. However, no further studies were carried out on this system because of the unknown toxicity of poly(4-vinylpyridine). This procedure is relatively easy and the kinetics of the polymerization process can be followed by spectral analysis. A possible disadvantage of this approach is the difficulty of removing the unreacted monomers before the preparation could be used clinically because of the possible toxicities (*Aliev et al., 1984; Ringsdorf and Schlarb, 1988*).

Attaching polymers to liposomes has not only the advantages of increased stability and possibly decreased leakage of entrapped solutes, but also the possibility of prolonging the circulation times of intravenously administered liposomes due to liposome surface modification, which reduces recognition by the RES. The Stealth® liposome originally utilized monosialoganglioside (G_{M1}) to achieve this effect (*Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Papahadjopoulos and Gabizon, 1990*). Subsequently, similar effects have been found when liposomes were coated with polysaccharides (*Sato and Sunamoto, 1992*) for targeting to tumors cells following intravenous administration.

A successful alternative to incorporating hydrophilic polymers has been the preparation of liposomes using PEGylated lipids, *i.e.*, liposomes having polyethylene glycol (PEG) chains that are chemically bonded to PE and extend out from liposome bilayer surfaces. Small liposomes with mean diameters ≤ 100 nm, in which PEG-PE had been incorporated, resulted in prolonged circulation times regardless of the bilayer fluidity (*Senior et al., 1991; Allen et al., 1991*) and reduced MPS uptake. The increase in tumor uptake of PEGylated liposomes was found to be approximately 25-fold compared to conventional liposomes. Inspite of increasing the blood circulation times of liposomes which are relatively stable with respect to leakage of entrapped solutes, only cells in the vascular compartment are accessible to liposome delivery. Transport of liposomes to extravascular tissues/spaces still remains a problem, even though the permeabilities of tumors or sites of infection are relatively higher, increasing the chances for liposomal drug delivery to these sites.

1.8. Microencapsulated Liposomes

Microencapsulation techniques have also been applied to liposomes. Nylon microcapsules of liposomes have been described (*Yeung and Nixon, 1988*; *Nixon and Yeung, 1989*). *Dong and Rogers (1993)* prepared liposome microcapsules by complex coacervation using acacia and gelatin. The liposomes are mixed with acacia-gelatin solution at 40°C which is then emulsified in an organic solvent to form a w/o emulsion. The temperature of the emulsion is adjusted to 10°C and the formed microcapsules are hardened with formaldehyde.

In all of these cases, the stability of liposomes was markedly increased and the leakage rate of the entrapped solutes was reduced considerably. *Kibat et al. (1990)* used poly-L(lysine)-encapsulated liposomes to demonstrate pulsed release of entrapped BSA from microencapsulated liposomes. The advantage of this method is the ability to successfully coat liposomes with polymers which do not have a specific functional group, however, precautions must be taken to avoid contact of the liposomes and the organic solvent.

C. Oral Administration of Liposomes

1.9. The GI Tract Environment

The chemical milieu that adversely affects the structural integrity of liposomes include low pH and the enzyme pepsin in the stomach, detergent action of bile salts, and the hydrolytic effects of phospholipases (A_1, A_2, C and D) and other degrading enzymes such as trypsin, amino and carboxypeptidases present mainly in the region of the duodenum. Hence, some peptide and non-peptide drugs can not be given orally due to their chemical instability and/or poor absorption from the GI tract, *e.g.* Insulin, AraC. Other macromolecules may be too large to be absorbed intact from the GI tract and, hence, show poor bioavailabilities.

1.9.1. Liposome Integrity

If a liposomal DDS is to be active orally, it must have properties that maintain liposome integrity and stability in the stomach and small intestine and deliver its contents to an absorption site and/or physically transport the entrapped solutes across the epithelial cells lining the ileum of the GI tract into the portal circulation or the lymphatics. So far conventional liposomes have not been able to meet these requirements and have been shown to be unstable in the presence of bile salts and phospholipases (*Richards and Gardener, 1978*).

Liposomes composed of phospholipids existing in the gel-state at physiologic temperature or which contain stabilizing lipids, such as CH and DCP, are more likely to retain their intergrity. Surface modification of liposomes using polymers could augment this behavior, and facilitate drug transport to achieve pharmacological levels. Polymeric modification of liposomes may also be used advantageously to control liposome fusion (*Sato and Sunamoto, 1992*) and aggregation tendencies (*e.g.*, due to Ca^{++} ions) (*Rowland and Woodley, 1980*).

1.9.2. Absorption of Liposomal Solutes

In addition to the liposome stability requirement, retention of liposomes preferably in the ileum would increase the possibility of liposome fusion or endocytosis with the enterocytes leading to transport of the entrapped solutes across the GI tract. There are a substantial number of reports wherein liposomes have been reported to increase the oral bioavailability of many solutes such as insulin, heparin and factor VIII (*Hashimoto and Kawada, 1979; Patel et al., 1982; Woodley, 1985; Petkowicz et al., 1989*). Only a few detailed studies have been carried out that suggest that conventional liposomes are not absorbed intact from the GI tract and play no specific role in facilitating the absorption of entrapped polar solutes (*Chiang and Weiner, 1987*). *Ariën et al. (1993)* have suggested that liposomes are probably broken down by the cytoplasmic enzymes present in enterocytes, so very few of the intact liposomes are observed in general circulation following oral administration of calcitonin-entrapped liposomes. It was concluded that leakage of the drug adjacent to the gut wall simply increased the concentration gradient of drug at absorption sites above that which it would have been following administration of free drug.

The same polymer that increases the stability of the liposomes could possess bioadhesive properties as well leading to increased residence time of the liposomes in the GI tract. Specifically, molecular labels, such as tomato lectins and rhodamine-B attached to the liposome surfaces, having high binding affinity to specific cells, *e.g.* in the ileum, would ensure concentration of liposomes at the sites of absorption. Such localization of liposomes in the vicinity of Peyer's patches has the additional advantage of complete particle uptake by the microfold cells (M-cells) overlying the Peyer's patches by endocytosis into the lymphatics, as shown for rhodamine-B-PE incorporated into DSPC liposomes (*Aramaki et al., 1993*). Research in this field is now focussing on acquiring a better understanding of the processes involved in the transport of materials across the GI tract epithelium, including the colon (*Tomizawa, 1993*).

1.10. Rationale for Coating Liposomes with Polysaccharides

Polysaccharide-coated liposomes have been found to be more stable than the conventional liposomes. *Sunamoto et al. (1983, 1985, 1987 and 1988)* have developed and employed naturally-occuring polysaccharides such as pullulan, dextran, amylopectin, levan, and mannan to coat liposomes. Such polysaccharide-coated liposomes show significant physicochemical stability in the systemic circulation, resistance to enzymatic destruction, and the ability to be targeted to specific organs and tumor cells (*Sato and Sunamoto, 1992*).

The rationale for coating liposomes with polysaccharide derivatives is to mimic cell membranes of bacterial or plant cells that have the ability to withstand hostile environmental conditions. Bacterial and plant cell membranes are enclosed by a cell wall composed mainly of polysaccharide derivatives. The cell wall is required to maintain the shape and stiffness of the cell and to protect the cell membranes against chemical and physicochemical stimuli such as changes in osmotic pressure, ionic strength, hydrogen ion concentration, and temperature, etc. On the other hand, the saccharide determinants, such as glycolipids and glycoproteins on cell surfaces, are considered to play an important role in various biological recognition processes including an antigen-antibody interaction, toxin recognition and cell-cell adhesion (*Sharon and Lis, 1981*). Studies on the interaction of liposomal membranes with derivatized polysaccharides have shown that naturally-occuring polysaccharides such as pullulan, dextran, mannan, amylose, amylopectin and levan interact strongly with the liposomal surfaces mostly by hydrophobic interaction (*Iwamoto and Sunamoto, 1982; Sunamoto et al., 1983 & 1984*).

1.11. Evaluation Methods and Requirements

In vivo studies are concerned with the effectiveness of liposome-entrapped drugs on biological responses. Since the results of such *in vivo* studies are often difficult to interpret, several *in vitro* experimental approaches have been employed in order to elucidate some of the mechanisms involved in the transport of solutes from mucosal to the serosal side of the gut wall. Three techniques have been used, namely diffusion cells, *in situ* perfusion technique, and the rat everted intestinal sac to evaluate the physical transport of liposomes.

The *diffusion cell* method has been widely used in transdermal studies (*Cooper and Patel, 1990*) but *Chiang (1986)* applied this technique to study liposome transport across intestinal membranes. In this method, a piece of intestinal segment is placed between the donor (mucosal) and a receiver (serosal) cell. Preparations of test components were applied to the mucosal compartment and samples are analyzed periodically from the serosal compartment. Because of the lack of an intact mesentry of the intestinal membrane, hence, transport is measured across the epithelium, muscular and connective tissues.

A more physiologic model known as modified *Doluisio technique* (*Doluisio et al., 1969; Park et al., 1984*) was used by *Schwinke et al. (1984)* to study liposome intestinal uptake. A section of the rat small intestine was cannulated with two L-shaped cannulae and syringes were attached to each end of the cannulae separately. The sample preparation was then introduced into the intestinal lumen, aliquots were withdrawn and analyzed periodically.

Wilson and Wiseman (1954) and later *Bridges et al. (1978)* evaluated the effect of uptake of liposome entrapped macromolecules using the *everted sac* of rat small intestine *in vitro*. *Rowland and Woodley (1981)* suggested that the uptake of liposome-entrapped ^{125}I-PVP into the serosal fluid was by absorptive endocytosis. *Woodley (1985)* compared the quantity of immunoreactive insulin that reached the serosal fluid of the everted sac with free and liposome-entrapped insulin and observed that liposome entrapment facilitated the transport of the intact hormone to the serosal side; 95% of the insulin was present within relatively intact liposomes. In contrast, no free drug was able to cross into the serosal compartment clearly demonstrating that the rat small intestine does have the capability of translocating liposomes from the mucosal to the serosal side. Since the uptake of liposomes could be by absorptive endocytosis, then it may be possible to enhance the efficiency of the uptake process by attachment of receptor-specific substrates to the outside surface of liposomes. The everted gut sac system has been a useful tool to study the mechanism of uptake of liposomes following oral administration.

The results of the *in vitro* studies strongly suggest that certain liposomes, *i.e.*, those which at 37°C are below the T_c temperature of their phospholipids, are more stable in the stomach and are capable of being endocytosed by the enterocytes of the small intestine. Hence, when gel state liposomes are used in oral delivery, they can potentially be endocytosed and transported across the gut wall. The fluid state liposomes, however, can interact better with brush border membranes but they are also more susceptible to intralumenal destruction and are unlikely to be absorbed intact (*Woodley, 1985*).

1.12. Aim

The overall aim of this research is to develop a functionalized liposome drug delivery system, for oral administration, such that it would facilitate the delivery of small molecules as well as macromolecules.

1.13. Hypothesis

Coating the liposomes with polysaccharide derivatives has the potential of improving their stability and drug retention capability until they or their entrapped solutes are absorbed.

1.14. Objectives

1. Select an appropriate naturally-occuring polysaccharide to coat liposomes, of a composition that encapsulates water-soluble drugs, and itself is not prone to destabilization under normal *in vitro* conditions,
2. Prepare the derivatized polysaccharides, O-Palmitoylpullulan and Cholesterolpullulan, which can anchor on liposome surfaces *via* their hydrophobic substituents,
3. Compare the behavior of liposomes, prepared from phospholipids in liquid-crystalline (DMPC/DOPC) and gel (DPPC) states, under simulated GI fluid conditions,
4. Encapsulate two model compounds, Cytosine-arabinoside (Cytarabine, AraC, mol. wt. 243.2 daltons) representing a small water-soluble non-peptide solute and a water-soluble peptide, ^{125}I-BP1-016 (23 amino acid residues, mol. wt. 2455 daltons), in different liposome formulations and determine liposome characteristics, such as, EE of entrapped solutes, solute-bilayer interaction, particle size distribution, liposome stability, and kinetics of release of entrapped solutes in simulated gastric and intestinal fluids USP at 37°C and,
5. Obtain physicochemical and microscopic information to describe and confirm polymer-bilayer interactions.

Table 1.1 Diseases in which liposomal drug delivery has shown considerable improvements (taken from *Lasic, 1993*)

FUNGAL	**PROTOZOAN**	**VIRAL**	**BACTERIAL**
Histoplasmosis (Amphotericin B)	Leishmaniasis (Antimonials)	HSV (Acyclovir)	Tuberculosis
Cryptococcoses (Amphotericin B)	Toxoplasmosis	AIDS (AZT)	Leprosy
	Malaria (Chloroquin)		Salmonellosis (Gentamicin)

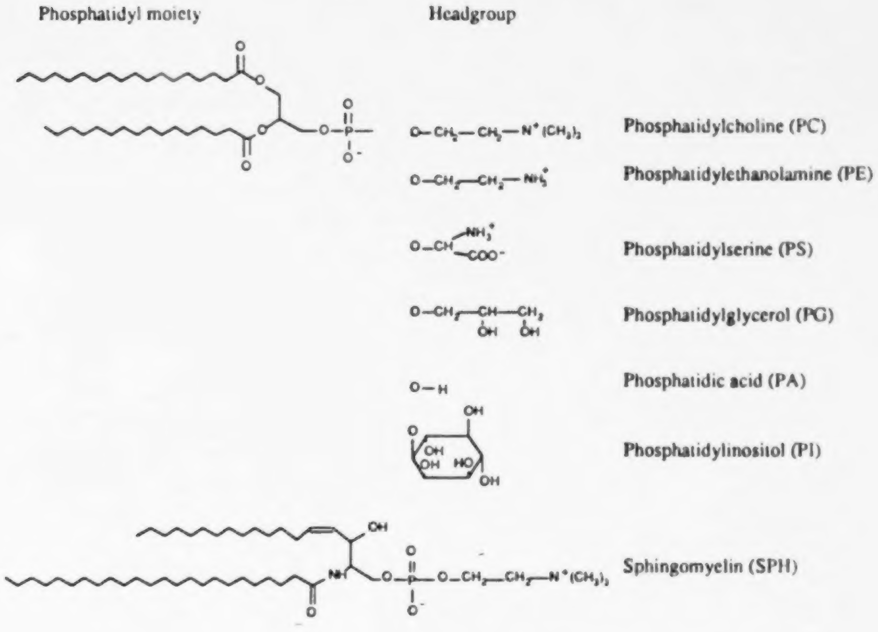
Phosphatidyl moiety
Headgroup
Phosphatidylcholine (PC)
Phosphatidylethanolamine (PE)
Phosphatidylserine (PS)
Phosphatidylglycerol (PG)
Phosphatidic acid (PA)
Phosphatidylinositol (PI)
Sphingomyelin (SPH)

Figure 1.1 Some common phospholipid molecular structures derived from 1,2-diacyl-*sn*-glycero-3-phosphate.

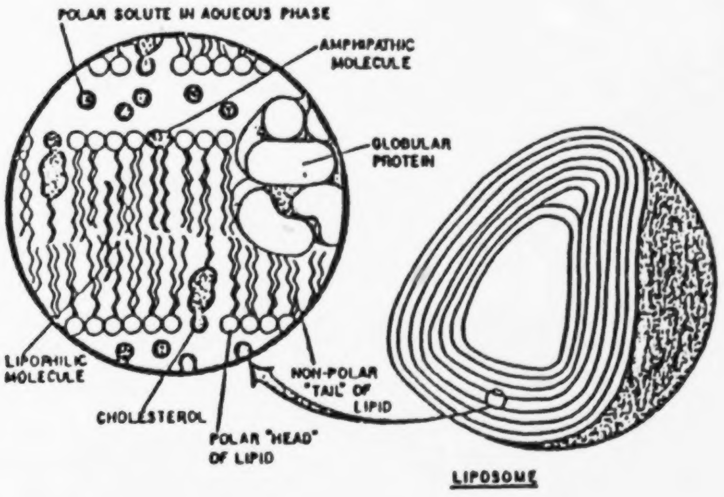
POLAR SOLUTE IN AQUEOUS PHASE
AMPHIPATHIC MOLECULE
GLOBULAR PROTEIN
LIPOPHILIC MOLECULE
CHOLESTEROL
POLAR "HEAD" OF LIPID
NON-POLAR "TAIL" OF LIPID
LIPOSOME

Figure 1.2 A multilamellar vesicle showing the sites of entrapment of cholesterol, proteins, polar, lipophilic, and amphipathic molecules.

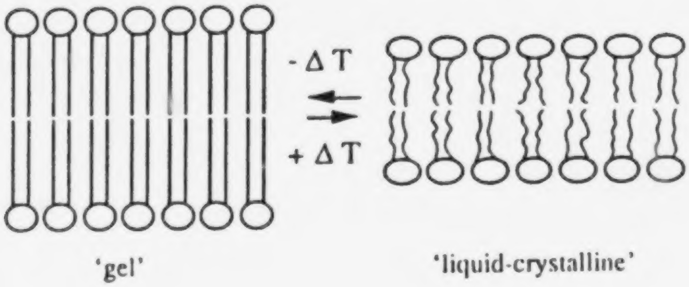
- ΔT
+ ΔT
'gel'
'liquid-crystalline'

Figure 1.3 Physical state of the phosphatidylcholine hydrocarbon chains in gel and liquid-crystalline states.

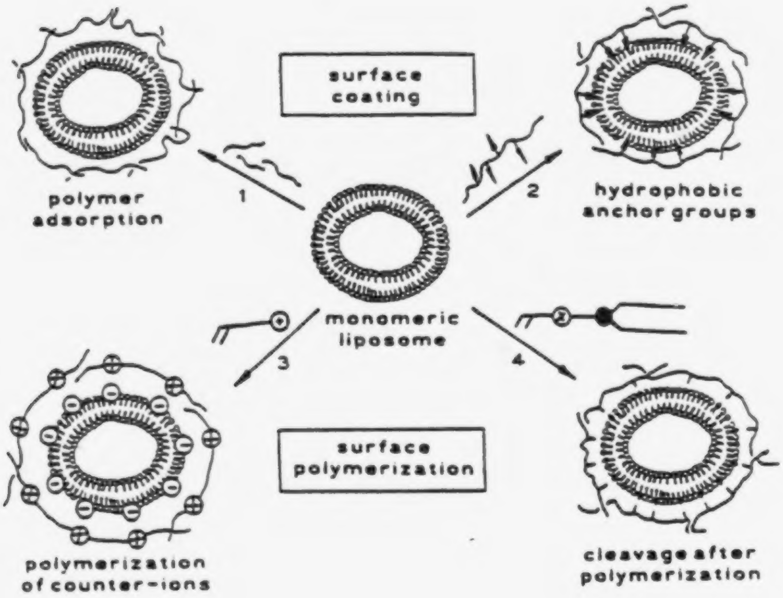
surface coating
polymer adsorption
1
2
hydrophobic anchor groups
monomeric liposome
3
4
surface polymerization
polymerization of counter-ions
cleavage after polymerization

Figure 1.4 Different strategies for stabilization of liposomes (*taken from Lasic, 1993*).

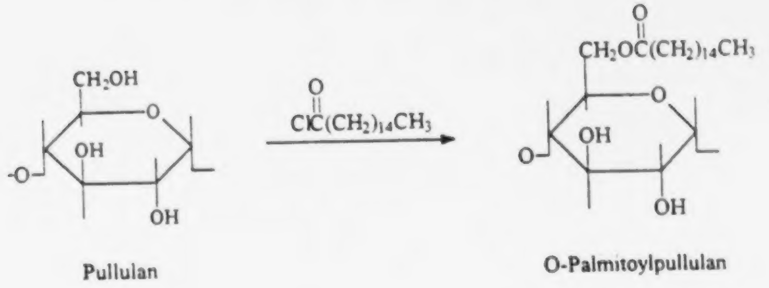
CH2OH
O
OH
-O
OH
Pullulan
O
ClC(CH2)14CH3
O
CH2OC(CH2)14CH3
O
OH
O
OH
O-Palmitoylpullulan

Figure 1.5 Synthesis of *O*-PalmitoylPullulan (OPP)

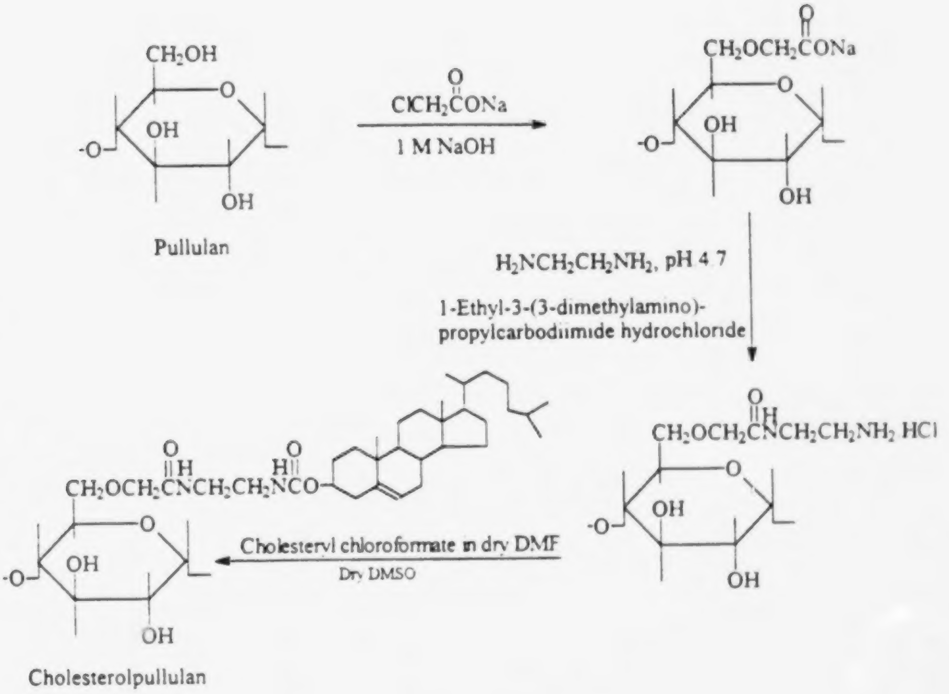
CH2OH
O
OH
-O
OH
Pullulan
O
ClCH2CONa
1 M NaOH
O
CH2OCH2CONa
O
OH
-O
OH
H2NCH2CH2NH2, pH 4.7
1-Ethyl-3-(3-dimethylamino)-
propylcarbodiimide hydrochloride
O
H
CH2OCH2CNCH2CH2NH2 HCl
O
OH
-O
OH
Cholesteryl chloroformate in dry DMF
Dry DMSO
O
H
H
O
CH2OCH2CNCH2CH2NCO
O
OH
-O
OH
Cholesterolpullulan

Figure 1.6 Synthesis of CholesterolPullulan (CHP)

1.15. References

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Chapter 2

POLYMER-COATED LIPOSOMES: IMPROVED LIPOSOME STABILITY AND RELEASE OF CYTOSINE ARABINOSIDE (AraC)

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2.1. Introduction

The microencapsulation of drugs offers several advantages in administration and therapy. One advantage is the ability to deliver small amounts of drug at a constant, or at least in a controlled manner, at sites of absorption to achieve the desired activity with a minimal amount of drug. Also, microencapsulation may protect the drug against harmful environmental influences, such as degrading enzymes or bile salts in the GI tract after oral administration, until the drug is actually released.

Liposomes have been reported to be stable in the GI tract if composed of distearoylphosphatidylcholine (DSPC) and cholesterol (*Woodley, 1985*) or if modified phospholipids are used which can be polymerized *in situ* to form a rigid, continuous bilayer. These types of formulations have not had much success as orally administered products because of unpredictable permeability behaviors and possible toxicities of reactants and products from polymerization. Alternatively, the formation of a polymeric membrane around the liposomes has some promise of counteracting the disruptive influences in the GI tract (*Rowland and Woodley, 1980*). Formulations of polysaccharide-coated liposomes have been prepared and tested *in vitro* for stability and release of ASA (*Dong and Rogers, 1991*). Earlier work (*Takada et al., 1984; Sunamoto and Iwamoto, 1985*) using derivatized polysaccharides to anchor the polymer to the surface of liposomes indicated reduced permeabilities of carboxyfluorescein, a water-soluble marker, and increased stabilities in the presence of phospholipase-D (*Sunamoto et al., 1983*).

The formulation of liposomes coated with polymers may also have application in controlling the rate of release of entrapped agents, particularly water-soluble solutes.

Cytarabine (AraC), a low molecular weight hydrophilic solute, was selected as a model to continue the development of polymer-coated liposome formulations. The stability and permeability of the liposome formulations exposed to different environmental conditions, as they would experience in the GI milieu following oral administration could be easily monitored.

2.2. Materials and Methods

2.2.1. Materials

L-α-dimyristoylphosphatidylcholine (DMPC, approx. 99%), L-α-dipalmitoyl-phosphatidylcholine (DPPC, approx. 99%), dicetyl phosphate (DCP), cholesterol (CH, >99%), pullulan, palmitoyl chloride and cytosine-ß-D-arabinofuranoside (AraC, mw 243.2 D) were used as received from Sigma Chemical Co. Sodium cholate (SC, Aldrich Chemical Co.) and all other chemicals were reagent grade. Water was demineralized and twice-distilled.

2.2.2. Synthesis of *O*-PalmitoylPullulan

Two grams of pullulan (average mol. wt. 54,000 Da) were dried *in vacuo* at 40°C for at least 3 h. The pullulan was then dissolved in 160 ml of anhydrous formamide and the contents stirred at 60°C in a water-bath for 2 h. Subsequently, 20 ml of anhydrous triethanolamine and 1 g of palmitoyl chloride were added and the contents stirred in a water bath at 60°C for 16 h. The reaction mixture was further diluted with 20 ml of water, then poured into 400 ml of methanol. After standing for five hours at 4°C, the precipitate was collected by filtration, washed with alcohol and dissolved in water. The translucent solution was treated by gel permeation chromatography (Sephadex G-50, 1.6x70 cm column), the fractions of derivatized polymer were pooled, and then lyophilized (Labconco freeze dryer) (*Hammerling and Westphal, 1967; Suzuki et al., 1977*). The degree of substitution of the polysaccharide was estimated from ^{1}H-NMR spectroscopic analysis of a solution of the product dissolved in DMSO-d_6 at room temperature (R.T., 23±2°C).

2.2.3. Preparation of Liposomes

Solutions of the lipids in chloroform were dried as a film in round-bottom flasks using a rotary evaporator. The flasks were flushed with N_2, then dried overnight in a vacuum desiccator at 40°C. Subsequently, the lipid film was hydrated with a solution of 30 mM AraC in pH 4.5, 5 mM potassium dihydrogen phosphate (PDP), yielding a final lipid concentration of 15 mM and a lipid:drug mole ratio of 1:2. Dispersion and swelling of the lipid were carried out at 12°C above the phase transition temperatures (T_m) of the phospholipids, *i.e.*, 35°C for DMPC composition and 54°C for DPPC composition. The dispersion was vortex-mixed for 8 min to form the liposomal suspension of multilamellar vesicles (MLVs), then subjected to five freeze-thaw (FT) cycles forming FATMLVs.

Polymer-coated liposomes were prepared by adding 1.5 ml of OPP solution in PDP to 3 ml of liposomal dispersion containing entrapped and free AraC, incubated at R.T. for at least 3 h, then at 4°C overnight. Uncoated liposomes were similarly treated but without OPP solution. In order to remove the untrapped drug and free polymer from the liposomal dispersion, the liposomes were ultracentrifuged (Beckman Model L8-55 ultracentrifuge, 150,000 x g, 15°C, 30 min), the supernatant removed, and the pellet resuspended with PDP solution.

2.2.4. Determination of Encapsulation Efficiency

Uncoated and OPP-coated liposomes (2 ml) were ultracentrifuged and washed with PDP solution as before. After centrifugation, the pellet obtained was dissolved in 100 ml of isopropanol:PDP solution (25:75 v/v). AraC was analyzed by reverse-phase HPLC at 273 nm (Novapak C_{18}, 15-cm column; mobile phase of 2 percent methanol in pH 4.5 PDP solution pumped at 1 ml/min) (*Kim and Howell, 1987*). Concentrations of AraC were determined from calibration curves (0-30 μg/ml) in either PDP solution only or in 25:75 v/v isopropanol:PDP solution ($r^2=1.00$, $n=12$ in both cases). The encapsulation efficiency (EE) was expressed as the ratio of the amount of AraC found in the pellet to the initial amount of AraC added.

2.2.5. Determination of OPP Coating Efficiency

Analysis of the amount of OPP which coated a liposome preparation employed a procedure that was reported previously for a polysaccharide (*Dong and Rogers, 1991*). Briefly, 1 ml of the OPP-coated liposomes were ultracentrifuged and the pellet was diluted to 10 ml with 1:1 v/v isopropanol:PDP solution. After 15 min of equilibration, absorbances were recorded at 400 nm and OPP concentrations were determined from a calibration curve. Absorbances were corrected for the presence of lipid after treating uncoated liposomes in a similar fashion. Coating efficiency (CE) was calculated as a ratio of the weight of OPP (mg) from coated liposomes to the weight of total lipid (mg) in liposomes (*Dong and Rogers, 1991*).

2.2.6. Particle Size Measurement

Samples of uncoated or OPP-coated liposomes (100 μl) were diluted to 3 ml with PDP solution, then subjected to laser light scattering (Brookhaven Instruments BI-90 Particle Sizer). The dust factor was maintained at 0.01 for all measurements.

2.2.7. Stability Studies of Liposomes

Uncoated or OPP-coated liposomes (4.5 ml) were ultracentrifuged as before, supernatants removed, and the pellet was resuspended in pH 5.6 buffer (0.017 M acetic acid and 0.157 M anhydrous sodium acetate) or pH 7.4 phosphate buffer solution (0.07M, Geigy Tables, 1970) to 7 ml. One ml samples were then mixed with different volumes of 20 mM SC solution and diluted to 10 ml with the respective buffer solutions. After a 15 min equilibration period, absorbances (turbidities) were measured spectrophotometrically at 400 nm (*Regen et al., 1980; Dong and Rogers, 1991*). Relative stabilities of the liposomes were determined as a function of the SC concentration from changes in the equilibrium turbidities.

2.2.8. Determination of Release Kinetics from Liposomes

Uncoated and OPP-coated FATMLVs (4.5 ml) were prepared as before except the pellet was resuspended to 9 ml with either Sorensen's pH 2.0 glycine/HCl buffer (Geigy Tables, 1970) or pH 7.4 phosphate buffer containing 10 mM SC solution (Richards and Gardner, 1978). The liposomes were incubated at 37°C (Dubnoff Metabolic Shaker), 250 μl samples were removed at various time intervals up to 24 h, centrifuged (14,000 rpm, 23°C, 4 min) and the supernatant analyzed for AraC by HPLC. The fraction of AraC released from liposomes was determined as,

$$\% \text{ Released} = (C-C_o)/(C_t-C_o) \times 100$$

where C_o is the initial drug concentration in the supernatant before incubation, C_t is the total drug concentration in the liposomes, and C is the concentration in the supernatant at time, t (*Dong and Rogers, 1991*).

2.3. Results and Discussion

The derivatization procedure employed to prepare OPP had a yield of approximately 15 percent and a degree of substitiution that was proportional to the concentration of palmitoyl chloride used in the reaction (*Sato, 1990*). As the degree of substitution of pullulan with the palmitoyl moiety increased, the aqueous solubility of the derivative decreased. Thus, it was necessary to obtain a derivative which had no solubility problem when used to coat liposomes. In this instance, a satisfactory degree of substitution was 1-2 palmitoyl chains per 100 saccharide units of pullulan. The ^{1}H-NMR spectrum of OPP yielded signals at 0.8 ppm and 1.2 ppm corresponding to the methyl and methylene protons of the palmitoyl chain, respectively. In contrast, these signals were absent in the ^{1}H-NMR of pullulan. This compares closely to the results previously reported by Sato (*1990*). Similarly derivatized polysaccharides have been used to prepare polymer-coated liposomes (*Sunamoto et al., 1983; Takada et al., 1984; Sunamoto and Iwamoto, 1985*) who reported that coating liposomes with hydrophobically-derivatized polysaccharides was necessary in order to minimize desorption of polysaccharide from liposome surfaces after dilution.

2.3.1. EEF of AraC in Liposomes

The EE of AraC in liposomes of various compositions are shown in Table 2.1. Generally, the EE was higher in DPPC than in DMPC or DSPC liposome compositions and increased with the addition of DCP. A phospholipid:CH mole ratio of 3:1 appeared to have the most favorable characteristics and maximum EE of 28 and 40 percent were obtained in DMPC:CH:DCP (3:1:2 mole ratio) and DPPC:CH:DCP (3:1:0.5 mole ratio) liposome compositions, respectively (Figure 2.1). It is also apparent in Figure 2.1 that the mechanisms of encapsulation were different for both liposome compositions. Even though AraC is greater than 99 percent ionized (pK_a = 9.7; *Newton and Kluza, 1978*) and DCP is 50 percent ionized (pk_a = 4.5; *Maitani et al., 1990*) at pH 4.5, maximum EE of AraC occurred in liposomes composed of DPPC:CH:DCP (3:1:0.5 mole ratio), and then remained constant at higher DCP contents. In comparison, EE of AraC in DMPC:CH:DCP liposomes increased proportionally with addition of DCP up to about 1.0 mole ratio, then plateaued between 1.0 and 2.0 mole ratio of DCP. Differences in fluidity of the liposomal bilayers and accessibility of ionized AraC molecules to negatively-charged binding sites are likely responsible for these observed behaviors. Liposomes of DPPC exist in the gel state (T_m = 41.5°C) at R.T. even at DPPC:CH (3:1 mole ratio) (*Demel and De Kruyff, 1976*). Increasing the mole ratio of DCP to 0.5 is unlikely to alter this state significantly. In contrast, liposomes of DMPC exist in the fluid state (T_m = 22°C) at R.T. even at DMPC:CH (3:1 mole ratio) (*Demel and De Kruyff, 1976*). Adding 2.0 mole ratio DCP could also increase fluidity of the bilayers (in addition to contributing a negative charge), and result in increased binding of AraC due to increased accessibility of the negatively-charged sites to positively-charged AraC molecules. Thus, the evidence suggests that higher EE of AraC in DPPC:CH:DCP liposomes arise mainly from increased volume of aqueous compartments in the liposomes due to electrical repulsion of the bilayers at pH 4.5 (*Westman et al., 1982; Mohr and Struve, 1991*). The rigid structure of the gel-state DPPC:CH:DCP liposomes hinders binding of AraC to DCP molecules in the bilayers. On the other hand, the more efficient electrostatic binding of AraC molecules to negatively-charged DCP molecules in fluid state bilayers (at pH 4.5) could account for the proportional uptake of AraC in the

DMPC liposome composition.

2.3.2. Coating Efficiency of Liposomes

Coating liposomes with a polymer is dependent on the degree of interaction between the polymer and the liposome surfaces. Many polymers such as dextran, pullulan or carboxylic acids are highly water-soluble and do not spontaneously coat dynamic liposome systems to produce stable films. Instead, a hydrophobic moiety is required to anchor the polymer chains to the bilayer surface (*Sunamoto and Iwamoto, 1985*). The coating efficiency of liposomes as a function of OPP concentration is shown in Figure 2.2. The coating efficiency of 15 mM of either DMPC:CH:DCP (3:1:2) or DPPC:CH:DCP (3:1:0.5) liposome compositions by OPP reached a maximum at an initial OPP concentration of 0.5 percent. The coating efficiency decreased at higher OPP concentrations, but the DMPC liposome composition also exhibited another maximum at 2.5 percent, indicating that OPP may also penetrate deeper into the bilayers at higher concentrations because of the fluid, liquid-crystalline state of DMPC liposomes at 23°C, and possibly due to polymeric adsorption on bilayer surfaces as multiple layers.

2.3.3. Effect of OPP-Coating on Particle Size

A comparison of the effect of polymer-coating on liposome size as determined by laser light scattering is given in Table 2.2. As expected, the DMPC:CH:DCP liposomes were smaller than DPPC:CH:DCP liposomes, but their sizes increased by 16 and 22 percent, respectively, as a result of coating with 0.5 percent OPP. Microscopic observation showed that the uncoated and OPP-coated liposomes did not aggregate and, in the latter case, liposomes were observed within polymeric shells. Coating the liposomes with OPP at pH 4.5 resulted in a decrease in the EE of AraC to 15 and 27 percent from 28 and 40 percent in DMPC and DPPC liposome compositions, respectively. Unencapsulated AraC was recovered in the supernatants after ultracentrifugation of the liposomal suspensions.

2.3.4. Stability of Liposomes in Sodium Cholate solutions

Relative stabilities of the two liposome compositions in SC solutions at pH 5.6 and 7.4 are depicted in Figures 2.3 and 2.4, respectively. The uncoated DPPC:CH:DCP liposome composition was observed to be more stable than the DMPC:CH:DCP composition at either pH. Furthermore, liposomes were considerably more stable at pH 7.4 than at 5.6. Since the cholic acid molecules are approximately 90 percent ionized at pH 7.4 (pK_a=6.4; *Merck Index, 1989*), they are much less effective in penetrating negatively-charged lipid bilayers (*O'Connor and Wallace, 1985*). At pH 5.6, the surface-active, unionized cholic acid molecules (~ 90 percent unionized) readily penetrate the lipid bilayers of the DMPC liposome composition. In comparison, the rigid gel-state DPPC liposomes resist unionized cholate penetration more than the fluid-state DMPC liposomes, resulting in their greater stability. Coating the liposomes with OPP improved the stability of the DMPC and DPPC compositions at pH 5.6 and the DMPC composition at pH 7.4. The stable DPPC liposome composition at pH 7.4 demonstrated no further improvement by coating with OPP.

2.3.5. Release of AraC from Liposomes

The release profile of AraC from each liposome composition at pH 2.0 and 37°C is depicted in Figure 2.5. Each is characterized by an initial rapid release phase for about 6 h followed by a slower release phase lasting for at least 24 h. Table 2.3 compares the fractions of AraC released during the rapid release phase of each liposome composition and the rate constants, k_o^u and k_o^c, derived from the slow release phases of uncoated and OPP-coated liposomes, respectively. It can be seen that a larger fraction of AraC was released during the rapid release phase from the DPPC than from the DMPC liposome composition but, thereafter, the rate of release was lower from the DPPC liposome composition. Coating liposomes with OPP yielded a smaller k_o, the magnitude of which is given by k_o^u/k_o^c. Thus, the OPP coating decreased the release rate by 2 times and almost 6 times for the DMPC and the DPPC liposome compositions, respectively. Similar behavior had been observed previously from measurements of the release of ASA from CM-chitin-coated DPPC liposomes at pH 5.6, 37°C (*Dong and Rogers, 1991*), and it had been concluded that the polymer altered the permeability of the liposome bilayers to the small ionized solute molecule rather than itself being a barrier to diffusion. The higher fraction of AraC released during the rapid release phase from the DPPC compared to the DMPC liposome composition when the liposomes were placed in pH 2.0 buffer solution is most likely due to the initial pH gradient effect on free AraC in the DPPC liposome composition (Figure 2.1) whereas AraC was mainly bound to the bilayers in the DMPC liposome composition.

Figure 2.6 illustrates the relative susceptibility to leakage of AraC from both liposome compositions in pH 7.4 phosphate buffer containing 10 mM SC at 37°C. Under these conditions, there is a larger initial release of drug from the uncoated DMPC (72 percent) versus DPPC (32 percent) liposome composition which is significantly reduced (to 55 percent) by coating the DMPC liposome composition with OPP but not the DPPC liposome composition (reduced to 28 percent). Thus, ionized cholate can readily penetrate the fluid-state liposomes to cause disruption and release of AraC, but not the gel-state liposomes. Consequently, the presence of OPP resulted in a 5-fold reduction in the release rate of AraC from DMPC:CH:DCP liposomes in 10 mM SC solution at 37°C from 6-24 h compared to 1.4-fold reduction from the DPPC:CH:DCP liposomes.

In summary, at pH 7.4 the stability of liposomes composed of DPPC:CH:DCP at 37°C was relatively high and the permeabilty to AraC was low even in the presence of up to 16 mM SC. Thus, little was gained by coating these liposomes with the polysaccharide. On the other hand, at pH 5.6 increasing SC concentrations destabilized these liposomes but coating with OPP significantly improved stability. In comparison, the stability of DMPC:CH:DCP liposomes was low at pH 7.4 and 5.6 and the permeability to AraC was relatively high in the presence of SC solution at pH 7.4. Coating these liposomes with polysaccharide markedly improved their properties in both instances. In the absence of SC at pH 2.0 (simulating pH conditions found in the stomach), the initial rapid release (0-6 h) of AraC was greater from the DPPC liposome composition than from the DMPC liposome composition, but thereafter it was slower in the former case, where coating by OPP also exerted a more pronounced reduction in the permeability.

Under the conditions of this study, it is concluded that DPPC:CH:DCP (3:1:0.5 mole ratio) liposomes could be an effective oral delivery system for a water-soluble solute such as AraC, if they reached that region of the GI tract intact which has a pH of 7-8. However, the environment of the stomach or duodenum could be deleterious to these liposomes if they are not protected. Coating liposomes of either composition with OPP appeared to have the potential of offering a significant level of protection against these influences. The same could be said of the DMPC:CH:DCP (3:1:2 mole ratio) liposomes but to a lesser degree. Further investigations underway involving phospholipases and other endogenous enzymes of the GI tract are intended to elucidate the relative susceptabilities of liposome compositions to these agents.

Table 2.1 Percent Encapsulation Efficiency of AraC in Liposome Compositions (FATMLVs) at pH 4.5[a]

Liposome Composition[b]	DMPC	DPPC	DSPC
CHOL (2:1)	2.3±0.1	4.4±0.9	1.5±0.8
CHOL:DCP (1.33:1:0.12)	11.7±1.5	27.4±2.5	19.4±2.4
CHOL:DCP (1.33:1:0.67)	20.3±2.3	33.4±1.7	-
CHOL:DCP (3:1:0.5)	16±0.9	40.2[c]±1.4	-
CHOL:DCP (3:1:2)	28[c]±2.7	40.1±1.5	-

[a] calculated as a fraction of initial AraC (30 mM) in 15 mM total lipid concentration, Means±SEM, n=6,

[b] numbers in brackets denote the phospholipid:lipid mole ratios,

[c] maximum EE of liposome compositions as a function of the DCP content (see Fig. 2.1).

Table 2.2 **Geometric Mean Diameters (nm) of Uncoated and OPP-coated Liposomes**

Type of Liposomes	DMPC:CHOL:DCP (3:1:2 mole ratio)	DPPC:CHOL:DCP (3:1:0.5 mole ratio)
Uncoated	760±23	856±18
OPP-coated	887±16	1048±33

Means±SEM, n=6.

Table 2.3 **Release Kinetics of AraC from Uncoated and OPP-coated Liposomes in pH 2.0 and 7.4 buffer mediums, 37°C**

Liposome Composition	% Ara-C Released After Rapid Release Phase[a]	k_o	k_o^u/k_o^c
pH 2.0 buffer			
DMPC:CH:DCP (uncoated)	22	0.48	
" (coated)	17	0.25	1.9
DPPC:CH:DCP (uncoated)	33	0.38	
" (coated)	27	0.07	5.7
pH 7.4 buffer containing 10 mM SC			
DMPC:CH:DCP (uncoated)	72	0.31	
" (coated)	55	0.06	5.1
DPPC:CH:DCP (uncoated)	32	0.23	
" (coated)	28	0.17	1.4

[a] after 6 h,

k_o^u and k_o^c represent the slopes of the constant release phases from 6-24 h of the uncoated (u) and OPP-coated (c) liposomes, respectively,

SC = sodium cholate.

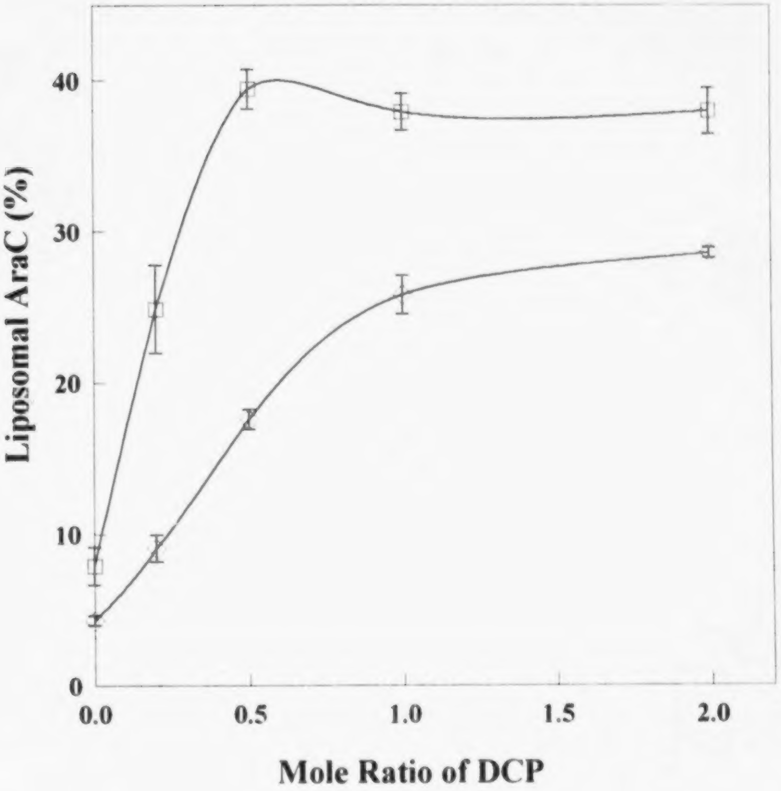

Figure 2.1 Encapsulation efficiency of Ara-C in DMPC:CHOL (◇) or DPPC:CHOL (□) (3:1 mole ratio) liposomes at pH 4.5 as a function of mole ratio of DCP. The total lipid concentration was 15 mM, and the initial Ara-C concentration was 30 mM. Means±SEM, n=4.

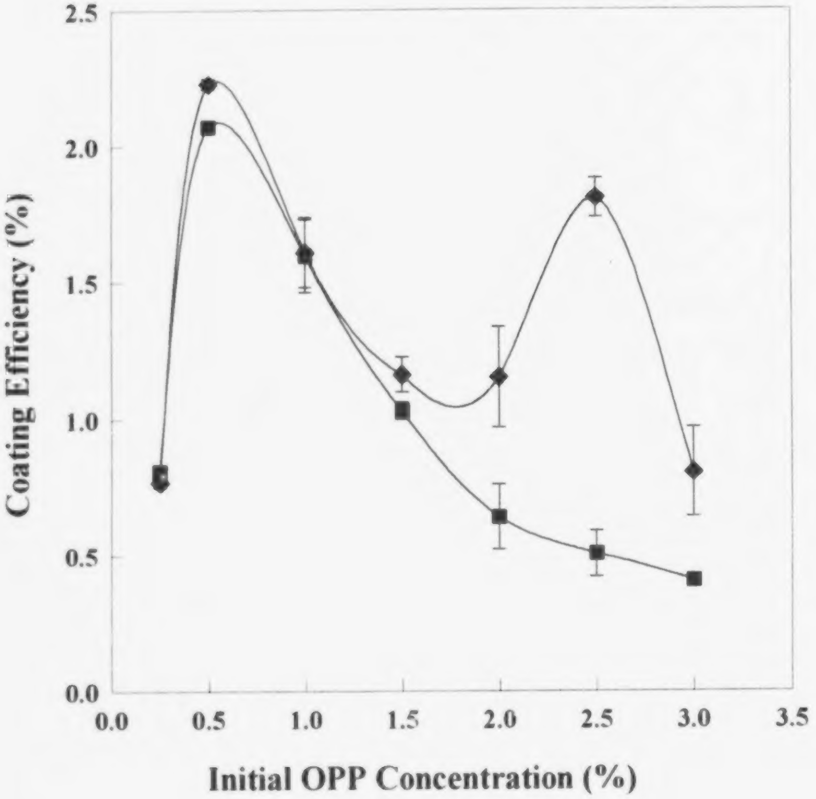

Figure 2.2 Coating efficiency (%) of liposomes by OPP as a function of the initial OPP concentration at pH 4.5. ◆, DMPC:CHOL:DCP; ■, DPPC:CHOL:DCP; Means±SEM, n=5.

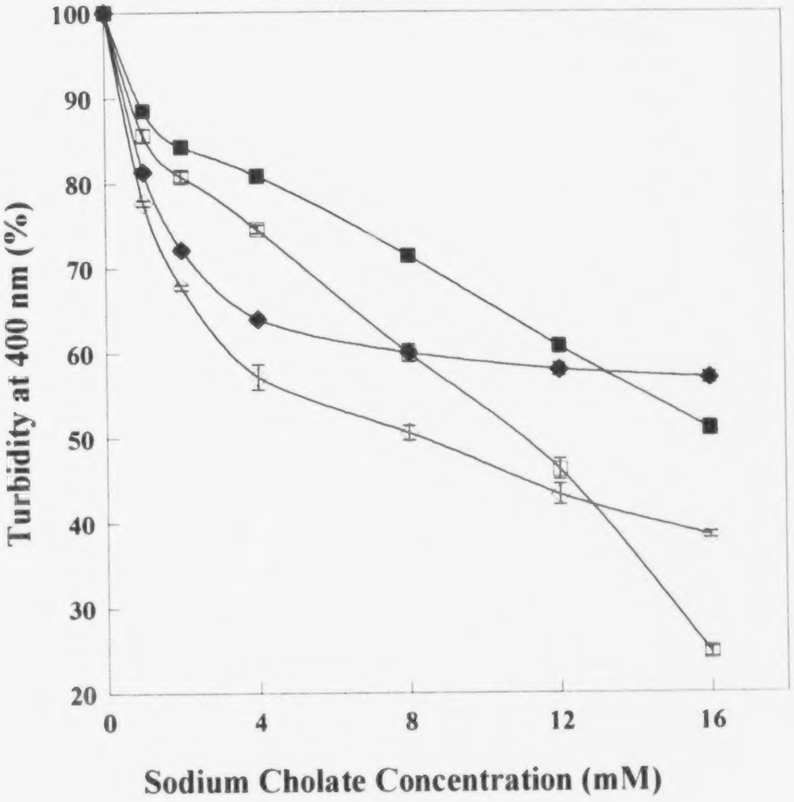

Figure 2.3 Stabilities (relative turbidities) of uncoated (open symbols) and OPP-coated (closed symbols) liposomes as a function of SC concentration in pH 5.6 acetate buffer at R.T. Liposomes were coated with an initial OPP concentration of 0.5 percent. ◇, ◆, DMPC:CHOL:DCP; □, ■, DPPC:CHOL:DCP; Means±SEM, n=4.

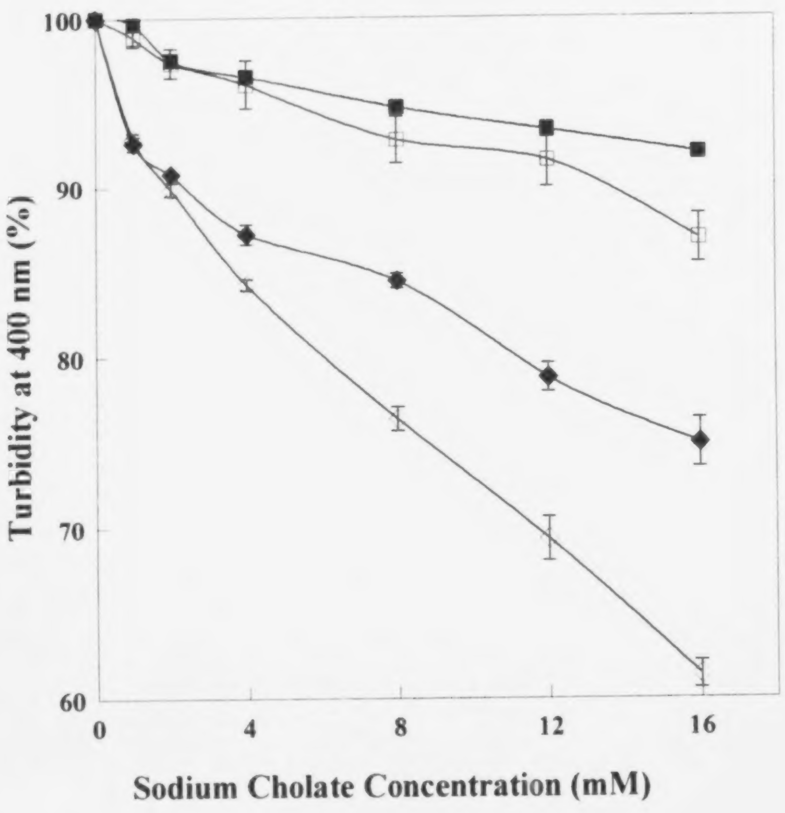

Figure 2.4 Stabilities (relative turbidities) of uncoated (open symbols) and OPP-coated (closed symbols) liposomes as a function of SC concentration in pH 7.4 phosphate buffer at R.T. Liposomes were coated with an initial OPP concentration of 0.5 percent. ◇, ◆, DMPC:CHOL:DCP; □, ■, DPPC:CHOL:DCP; Means±SEM, n=4.

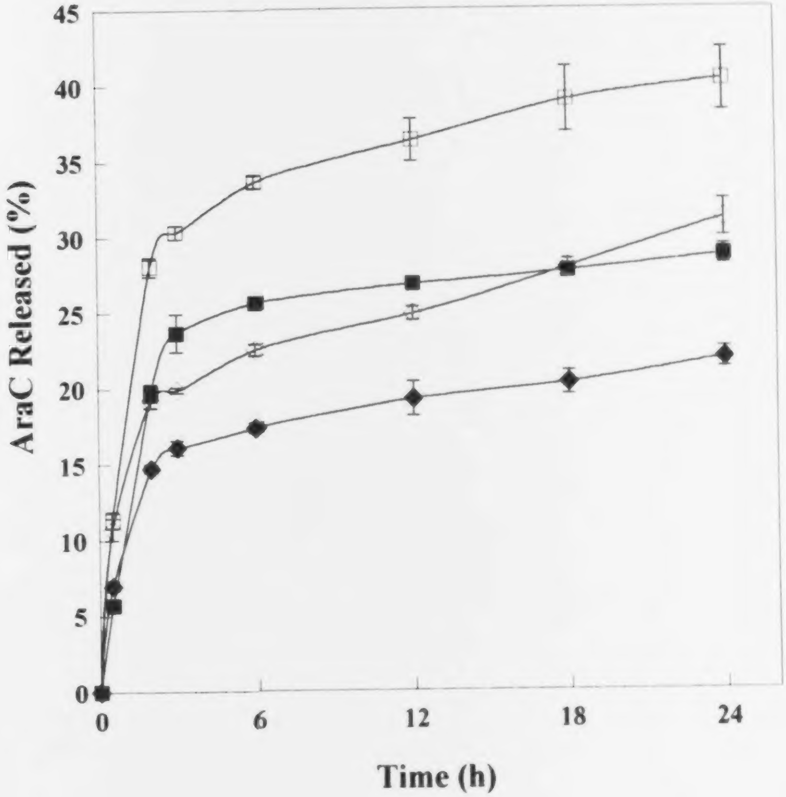

Figure 2.5 Release kinetics of Ara-C from uncoated (open symbols) and OPP-coated (closed symbols) liposomes at pH 2.0, and 37°C. ◇, ◆, DMPC:CHOL:DCP; □, ■, DPPC:CHOL:DCP; Means±SEM, n=3 .

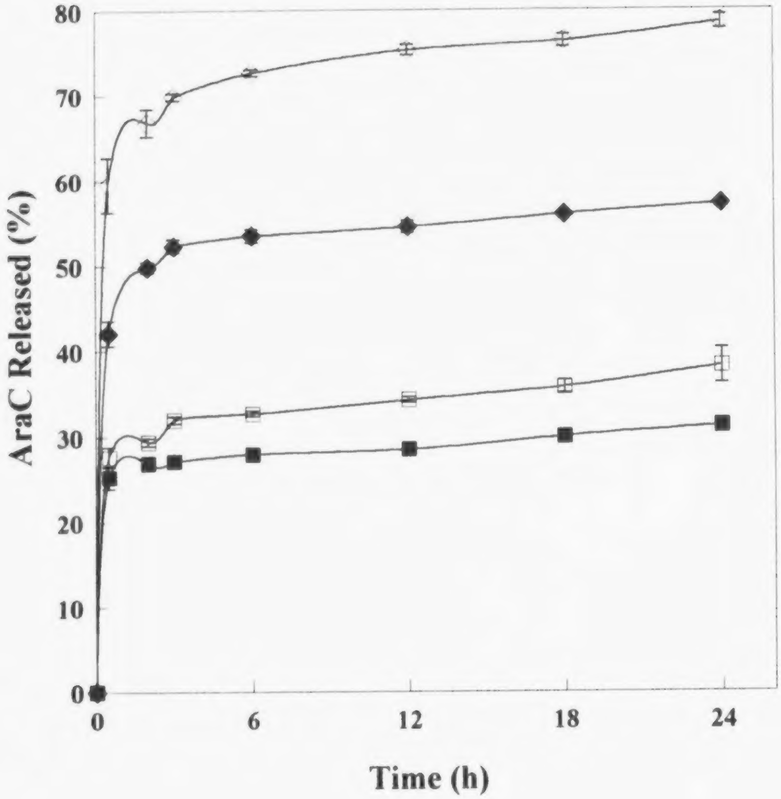

Figure 2.6 Release kinetics of Ara-C from uncoated (open symbols) and OPP-coated (closed symbols) liposomes in 10 mM SC solution at pH 7.4 and 37ºC. ◇, ◆, DMPC:CHOL:DCP; □, ■, DPPC:CHOL:DCP; Means±SEM, n=3.

2.4. References

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Chapter 3

DECREASED RELEASE OF CYTARABINE FROM PULLULAN-COATED LIPOSOMES IN SIMULATED GASTRIC AND INTESTINAL FLUIDS

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3.1. Introduction

The potential of liposomes as a drug delivery system is being investigated by many researchers. Liposomes have been successfully administered by various routes, including parenteral (*Fidler et al., 1981; Woodle et al., 1992*), ophthalmic (*Niesman, 1992*), inhalation (*Kellaway and Farr; 1990; Niven et al., 1991; Martin, 1990*), and transdermal (*Egbaria and Weiner, 1991; Lasch and Wohlrab, 1986; Lasch et al., 1991*) and have delivered small lipophilic or hydrophilic molecules as well as proteins and peptides (*Fidler et al., 1981; Woodle et al., 1992*). However, the same degree of success has not been obtained when liposomes have been tested by the oral route (*Chiang and Weiner, 1987; Sunamoto et al., 1990*). One of the main problems of the oral route is the instability of liposomes in the hostile environment of the GI tract, particularly in the lower intestinal regions. Another problem is their low affinity for the epithelial surface of the GI tract. Consequently, the residence time of liposomes is fairly short, being carried along easily by the mucous secretions.

In recent years, there have been several approaches towards encasing liposomes with polymers and these have shown to possess increased stability (*Sunamoto et al., 1990; Sunamoto and Iwamoto, 1985*). Very few attempts have been made, however, to develop these modified liposomes for oral use. In view of this, work in our laboratory has been aimed at using this approach of polymer-coated liposomes to improve liposome stability and decrease the leakage of entrapped water-soluble solutes (*Dong and Rogers, 1991; Dong and Rogers; 1992; Dong and Rogers, 1993*). This report describes a liposome formulation approach modelled after *Sunamoto et al. (1985)* using a derivatized polysaccharide as a surface coating, its effect on the physical properties of the liposome, and its role in altering the permeability of the liposome to a small water-soluble model drug. The results of this study could have important ramifications in the development of liposomes for oral drug delivery.

3.2. Materials and Methods

3.2.1. Materials

L-α-dimyristoylphosphatidylcholine(>99%),L-α-dipalmitoylphosphatidylcholine (>99%), dicetylphosphate (DCP), cholesterol (CH, >99%), pullulan (average mol. wt. 54,000 daltons), palmitoylchloride and cytosine-ß-D-arabinofuranoside (AraC) were used as received from Sigma Chemical Co., St. Louis, MO. Potassium dihydrogen phosphate (PDP) and isopropanol were obtained from BDH Inc., Toronto, Canada. Water was demineralized and twice-distilled before use.

3.2.2. Synthesis of O-PalmitoylPullulan

Two grams of pullulan were dried *in vacuo* at 40°C for at least 3 h. The polysaccharide was then dissolved in 160 ml of anhydrous formamide and the contents stirred at 60°C in a water-bath for 2 h. Subsequently, 20 ml of anhydrous triethanolamine and 1 g of palmitoyl chloride were added and the contents stirred in a water bath at 60°C for 16 h. The reaction mixture was diluted with 20 ml of water, then added to 400 ml of methanol. After standing for at least five hours at 4°C, the precipitate was collected by filtration, washed with alcohol and dissolved in water. The translucent solution was processed by gel permeation chromatography (Sephadex G-50, 1.6x70 cm column), the fractions of derivatized polymer were pooled, and then lyophilized (Labconco freeze-dryer) (*Hammerling and Westphal, 1967; Suzuki et al., 1977*). The degree of substitution of the polysaccharide was estimated from ^{1}H-NMR spectroscopic analysis of a solution of the product dissolved in DMSO-d_6 at room temperature (23 ± 2°C).

3.2.3. Preparation of Liposomes

Solutions of the lipids in chloroform were evaporated (rotary evaporator) forming films on the walls of round-bottom flasks. The flasks were flushed with nitrogen gas, then dried overnight in a vacuum desiccator at 40°C. Subsequently, the lipid film was hydrated with a solution of 30 mM AraC in 5 mM, pH 4.5 PDP solution yielding a final lipid concentration of 15 mM and a lipid:drug mole ratio of 1:2. Dispersion and swelling of the lipid were carried out at about 12°C above the phase transition temperatures (T_m) of the respective phospholipids. The dispersion was vortex-mixed for 8 min to form the liposomal suspension of multilamellar vesicles (MLVs), then subjected to five freeze-thaw (FT) cycles forming FATMLVs.

Polysaccharide-coated liposomes were prepared by adding 1.5 ml of OPP solution in PDP to 3 ml of liposomal suspension followed by intermittent stirring and equilibration for a minimum of 3 h, then stored overnight in the refrigerator at 4°C. Uncoated liposomes were similarly treated. The liposomes were ultracentrifuged (Beckman Model L8-55 ultracentrifuge, 150,000 x g, 15°C, 30 min) and the supernatant containing free drug and polymer was removed. The pellets were re-suspended with release medium at the beginning of an experiment.

3.2.4. Determination of Encapsulation Efficiency

Uncoated and OPP-coated liposomes (2 ml) were ultracentrifuged and washed with PDP solution, then the pellet was dissolved in 100 ml of isopropanol:PDP (25:75 v/v) solution. AraC was analyzed by reverse-phase HPLC at 273 nm (Novapak C_{18}, 15-cm column; mobile phase of 2 percent methanol in pH 4.5 PDP solution, at a pump rate of 1 ml/min) (*Kim and Howell, 1987*). Concentrations of AraC were determined from calibration curves (0-30 μg/ml) in either PDP solution only or in 25:75 v/v isopropanol:PDP system ($r^2=1.00$, n=12 in both cases). EE was expressed as the ratio of the amount of AraC analyzed in the pellet to the initial amount of AraC added to the original liposome preparation.

3.2.5. Determination of OPP Coating Efficiency

The method of analysis of the amount of OPP which coated a liposome preparation followed a procedure that was reported previously for a polysaccharide (*Dong and Rogers, 1991*). Briefly, 1 ml of the OPP-coated liposomes were ultracentrifuged and the pellet was diluted to 10 ml with 1:1 v/v isopropanol:PDP solution. After 15 min, absorbances were recorded at 400 nm and the OPP concentration determined from a calibration curve. Absorbances were corrected for the presence of lipid by similarly treating uncoated liposomes. Coating efficiency was calculated as a ratio of the weight (mg) of OPP in OPP-coated liposomes to the weight (mg) of lipids in the liposome suspension.

3.2.6. Particle Sizing and Optical/Electron Microscopy Studies

Uncoated and OPP-coated liposomes (100 μl) were sized by laser light scattering (Brookhaven Instruments BI-90 Particle Sizer) after diluting 100 μl to 3 ml with PDP solution. The dust factor was maintained at 0.01 in each case.

Uncoated and OPP-coated liposomes of both DMPC and DPPC compositions were observed under an optical microscope (Olympus, Model 219738) at x1000 magnification. Also, liposomes were freeze-fractured to observe the effect of coating by OPP using transmission electron microscopy (TEM).

3.2.7. Differential Scanning Calorimetry Studies

A high-sensitivity differential scanning calorimeter (Hart Scientific, DSC, Model 4207, Pleasant Grove, UT) was employed to evaluate changes in liposome thermotropic phase behavior as a result of AraC and OPP-lipid interactions. Phospholipid dispersions (20 mg/ml) of DPPC and DPPC:CH:DCP compositions with and without AraC and OPP were analyzed. Samples were scanned between 10 and 90°C at a scan rate of 15°C/hr for both heating and cooling scan modes. At least three scans were carried out to ensure equilibrium and reproducibility.

3.2.8. Determination of AraC Release Kinetics in Simulated GI Fluids

OPP-coated and uncoated FATMLVs (4.5 ml) were prepared as before, except the pellet was resuspended to 25 ml in simulated gastric (SG) or simulated intestinal (SI) fluid USP and the liposome suspensions were incubated at 37°C. (Dubnoff Metabolic Shaker), Samples of 250 μl were removed at various time intervals for 12 h, centrifuged (14,000 rpm, 23°C, 4 min) (Eppendorf centrifuge 5415) and AraC was analyzed in the supernatant by HPLC. The fraction of AraC released from liposomes, under sink conditions, was determined from,

$$\% \text{ Released} = (C-C_o)/(C_t-C_o) \times 100$$

where C_o is the initial drug concentration in the supernatant before incubation, C_t is the total drug concentration in the liposomes, and C is the concentration in the supernatant at time, t. Statistical comparisons between the fraction of AraC released from uncoated and OPP-coated liposomes were made using one-way ANOVA ($\alpha = 0.05$).

3.3. Results and Discussion

The derivatization procedure employed to prepare OPP gave a yield of approximately 15 percent and a degree of substitution that was proportional to the concentration of palmitoyl chloride used in the reaction. As the degree of substitution of pullulan with the palmitoyl moiety increased, the aqueous solubility of the derivative decreased. Thus, it was necessary to obtain a derivative which had no solubility constraints when used to coat liposomes. In this instance, a satisfactory degree of substitution was 1-2 palmitoyl chains per 100 saccharide units of pullulan. The ^{1}H-NMR spectrum of OPP yielded peak signals at 0.8 ppm and 1.2 ppm corresponding to the methyl and methylene protons of the palmitoyl chain, respectively. In contrast, these signals were absent in the ^{1}H-NMR of pullulan. This compares closely to results reported previously (*Sato 1990*).

3.3.1. Encapsulation Efficiency and Size Distribution

Using FATMLVs, the encapsulation efficiencies of AraC reached 28 and 40 percent in liposomes of DMPC and DPPC compositions, respectively (data obtained from chapter 2). However, when liposomes were coated from a solution of 0.5 percent OPP, representing the maximum coating efficiency, the EEs were reduced to 15 and 27 percent. Preliminary trials had indicated that the higher levels of entrapment of AraC in DPPC:CH:DCP liposomes were due to a portion of the AraC unbound entrapped in the aqueous compartments, the remaining being electrostatically bound to the negatively-charged bilayers, whereas the AraC in DMPC:CH:DCP liposomes was mainly bound. Thus, it appears that anchoring OPP to the surfaces of the liposomes displaced AraC from binding sites in both cases.

There is a question of whether OPP added to the liposomes alters the particle size distribution which may influence the release kinetics of AraC. The linearity of the results of log probability vs. size plots in Figures 3.1 and 3.2 confirm a normal distribution for both uncoated and polymer-coated liposomes. No evidence of aggregation was found during the measurements. Furthermore, it is apparent that the average size of OPP-coated liposomes was larger than uncoated liposomes, suggesting that the polymer formed a layer having a characteristic thickness at the bilayer surfaces.

3.3.2. Microscopic Examination of Liposomes

Microscopic evidence supported the argument of a uniform coat of polymer surrounding the liposomes. Uncoated liposomes viewed with the optical microscope appeared as transparent, spherical vesicles whereas OPP-coated liposomes appeared translucent with noticeable wall material surrounding them. In some cases, several liposomes could be observed within a shell of polymer.

Transmission electron micrographs (TEM) of freeze-fractured liposomes (Figures 3.3 and 3.4) provide further information of liposome characteristics and evidence of association of polymer. Coated liposomes are clearly distinguishable from uncoated liposomes where an outer skin of the coated liposomes can be seen. Small convex indentations, suggesting phase separation, are also apparent in TEMs of uncoated liposomes but not the polymer-coated liposomes.

3.3.3. Characterization of Multicomponent DPPC Liposomes

DSC thermotropic behavior of liposomes from DMPC and DPPC compositions were studied. Only those of DPPC liposomes are depicted in Figure 3.5. An endothermic peak at 41.5°C, representing its T_m with a pre-transition peak at 38°C, is characteristic of DPPC (curve 1) (*McMullen et al., 1993; Lewis et al., 1987*). In comparison, the thermogram of DPPC:CH:DCP liposome composition is broadened and a major shift in T_m occurred (curve 2), indicating a reduced enthalpy and cooperativity of the host phospholipid bilayer main phase transition, similar to the observation of others (*McMullen et al., 1993*). The incorporation of AraC of which a portion binds to DCP, hardly influenced the physical state of the liposomal bilayers (curve 3). In contrast, coating the liposomes with OPP significantly increased the cooperativity in the bilayers, suggesting that the polymer attachment at the liposome surfaces restricted movement of the bilayer molecules (curve 4). Coating the liposomes containing AraC with OPP appears to have a lower cooperativity in the bilayers than in the absence of AraC (curve 5) even though this resulted in a lower EE (Table 3.1). This may be due to a shifting of CH molecules deeper into the bilayer because of the restriction at the surfaces, which because of its rigid sterol ring, increases the disruption and the cooperativity among the hydrocarbon chains.

3.3.4. Kinetics of Release of AraC from Liposomes

There are two distinctly different environments in which liposomes must survive if they are going to be successful as an oral drug delivery system, namely, gastric fluid and intestinal fluid conditions. Thus, the results of release of AraC from DMPC and DPPC liposome compositions under these simulated conditions are shown in Figures 3.6 and 3.7. The release profiles are characterized by an initial rapid diffusion of AraC from the liposomes after mixing the liposomes with the medium followed by a slower rate, after about 2 h, which followed first-order kinetics and which lasted for at least 12 h. In SG fluid (Figure 3.6), approximately 55 percent of AraC was released from uncoated liposomes in 2 h whereas only about 40 percent was released from OPP-coated liposomes. The first-order rate constant, k_1, determined from 2-12 h was also significantly lower ($p<0.05$) from OPP-coated liposomes (Table 3.2). The ratio, k_1^u/k_1^c, corresponding to the rate constants of uncoated and coated liposomes was calculated to be 1.2 and 1.6 for DMPC and DPPC liposome compositions, respectively. Thus, coating liposomes with OPP reduced the leakage rate of entrapped AraC by 20 and 60 percent, respectively. As a result, about 45 percent of the encapsulated amount of AraC still remained after 12 h in OPP-coated liposomes. Although the kinetics of release were less from coated liposomes, there was no significant difference between the liposome compositions in SG fluid in either the coated or the uncoated state.

In SI fluid, the initial rapid release of AraC from uncoated liposomes of DPPC composition was 15 percent less than from liposomes of DMPC composition as can be seen in Figure 3.7. This difference, compared to no difference in SG fluid, is likely due to the influence of pH on the ionization of AraC and the fluidity of the bilayers. Exposing the liposomes to a pH 7.4 medium, at which AraC is less ionized, should cause less binding to the bilayers resulting in some leakage from the liposome, particularly from the outer bilayers. Liposomes of DMPC composition exist in the fluid, liquid crystalline state at 37°C (T_m = 23°C), and generally these have higher permeabilities than liposomes of DPPC composition which exist in the gel state at this temperature.

The release of AraC from OPP-coated DPPC:CH:DCP liposomes in SI fluid after 2 h was only 20 percent, compared to 47 percent from DMPC:CH:DCP liposomes (Table 3.2). Thereafter, first order release proceeded at a significantly slower rate ($p<0.05$) and the ratio, k_1^u/k_1^c, was 2.5 and 1.6 for liposomes of DMPC and DPPC compositions, respectively. This represents a release rate reduction of 150 and 60 percent from OPP-coated DMPC:CH:DCP and DPPC:CH:DCP liposomes, respectively. Consequently, 63 percent of encapsulated AraC still remained in DPPC:CH:DCP liposomes compared to 45 percent in DMPC:CH:DCP liposomes, after 12 h.

In summary, the encapsulation and release of the small, water-soluble model solute, AraC, have been found to be determined mainly by the liposome composition and the pH of the release medium. Gel-state liposomes allowed higher EEs and significantly slower release rates than liposomes in the liquid-crytalline state, even though some AraC was electrostatically bound to their bilayers. The stable adsorption of OPP on liposome compositions significantly reduced the initial amount of AraC released, and the release rate during 2 - 12 h period. The main mechanism of polymer stabilization appears to be by restriction of movement of the surface molecules by OPP with increased ordering (*i.e.* increased cooperativity). In SG fluid, AraC was released from uncoated liposomes at a fairly high rate, but rates were reduced substantially by coating with OPP. In SI fluid, coating liposomes with OPP again caused a reduction in the rate of release of AraC, reducing the fraction released from DPPC:CH:DCP liposomes to only 36 percent after 12 h. This may be adequate to consider OPP-coated liposomes to deliver drugs by the oral route.

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Table 3.1 Percent Encapsulation Efficiency of AraC in Uncoated and OPP-coated Liposomes at pH 4.5[a]

Liposome Composition[b]	Uncoated[c]	OPP-coated
DMPC:CHOL:DCP (3:1:2)	28±2.7	15±2.5
DPPC:CHOL:DCP (3:1:0.5)	40.2±1.4	27±1.9

[a] calculated as the fraction of initial AraC concentration (30 mM),

[b] numbers in brackets are the mole ratios of the lipid components, the total lipid concentration was 15 mM,

[c] taken from Table 2.1.

Table 3.2 **Release of AraC from Uncoated and OPP-coated DMPC:CH:DCP and DPPC:CH:DCP Liposomes in SG and SI fluids USP, 37°C**

Release Medium	Liposome Type	% AraC Released at 2 h	12 h	k_1 (h^{-1})	k_1^u/k_1^c
SG, pH 1.2	Uncoated DMPC[a]	57	75	4.3±0.3	
	OPP-coated DMPC	38	55	3.6±0.3	1.2
	Uncoated DPPC[b]	53	81	6.4±0.7	
	OPP-coated DPPC	37	57	4.0±0.2	1.6
SI, pH 7.4	Uncoated DMPC	57	78	4.8±0.2	
	OPP-coated DMPC	47	55	1.9±0.2	2.5
	Uncoated DPPC	41	65	5.3±0.6	
	OPP-coated DPPC	20	37	3.4±0.5	1.6

[a] DMPC:CH:DCP (3:1:2 mole ratio) composition,

[b] DPPC:CH:DCP (3:1:0.5 mole ratio) composition,

k_1^u and k_1^c were derived from the slopes of 2-12 h release phase from uncoated and OPP-coated liposomes, respectively,

Means±SEM, n=3.

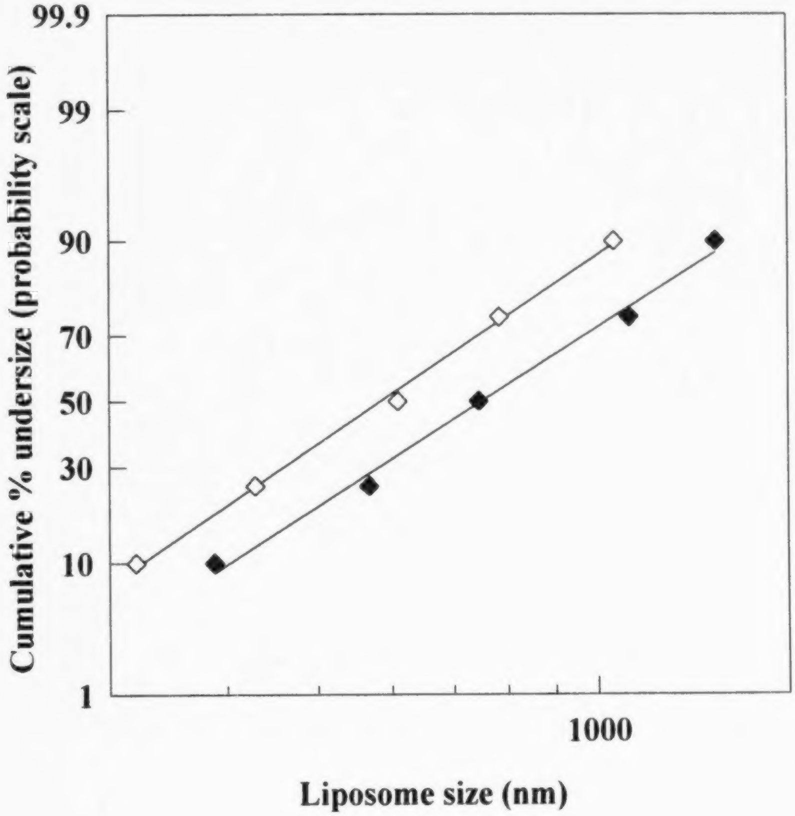

Figure 3.1 Log-probability plots of sizes of DMPC:CH:DCP (3:1:2 mole ratio) uncoated (open symbols) and OPP-coated (closed symbols) liposomes.

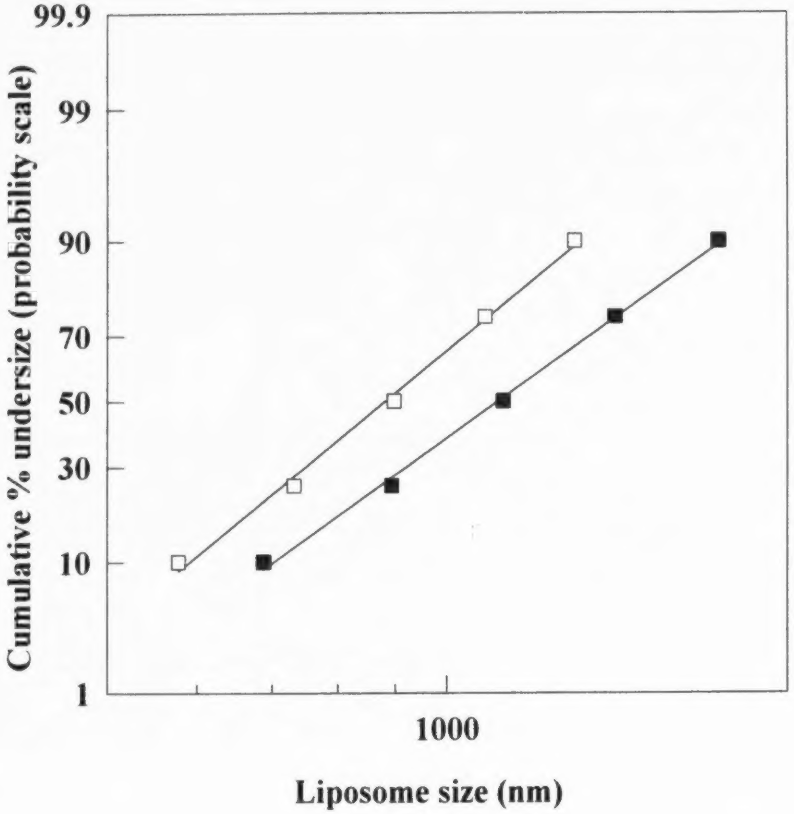

Figure 3.2 Log-probability plots of sizes of DPPC:CH:DCP (3:1:0.5 mole ratio) uncoated (open symbols) and OPP-coated (closed symbols) liposomes.

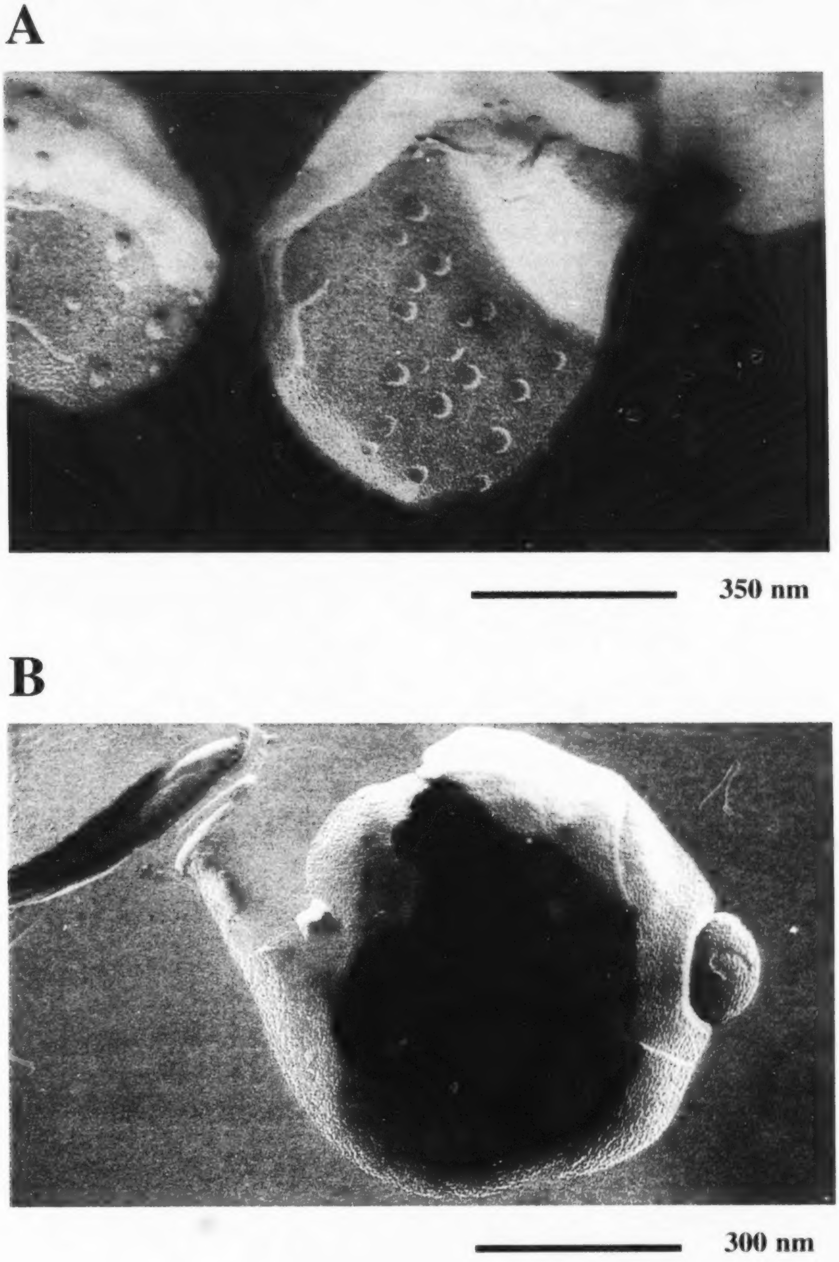
A
350 nm
B
300 nm

Figure 3.3. Transmission electron micrographs of DMPC:CH:DCP (3:1:2 mole ratio) freeze-fractured liposomes. **(A)** Uncoated; **(B)** OPP-coated.

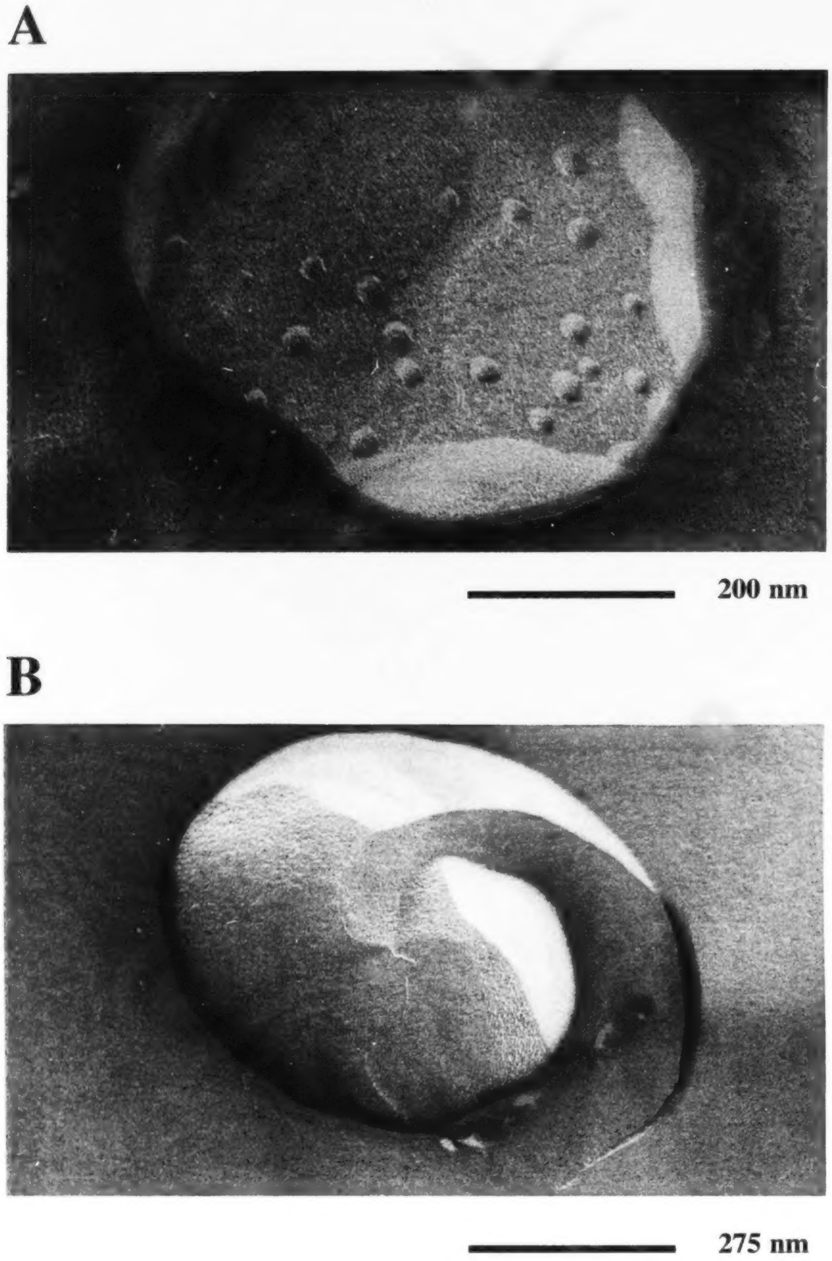
A
200 nm
B
275 nm

Figure 3.4. Transmission electron micrographs of DPPC:CH:DCP (3:1:0.5 mole ratio) freeze-fractured liposomes. (**A**) Uncoated; (**B**) OPP-coated.

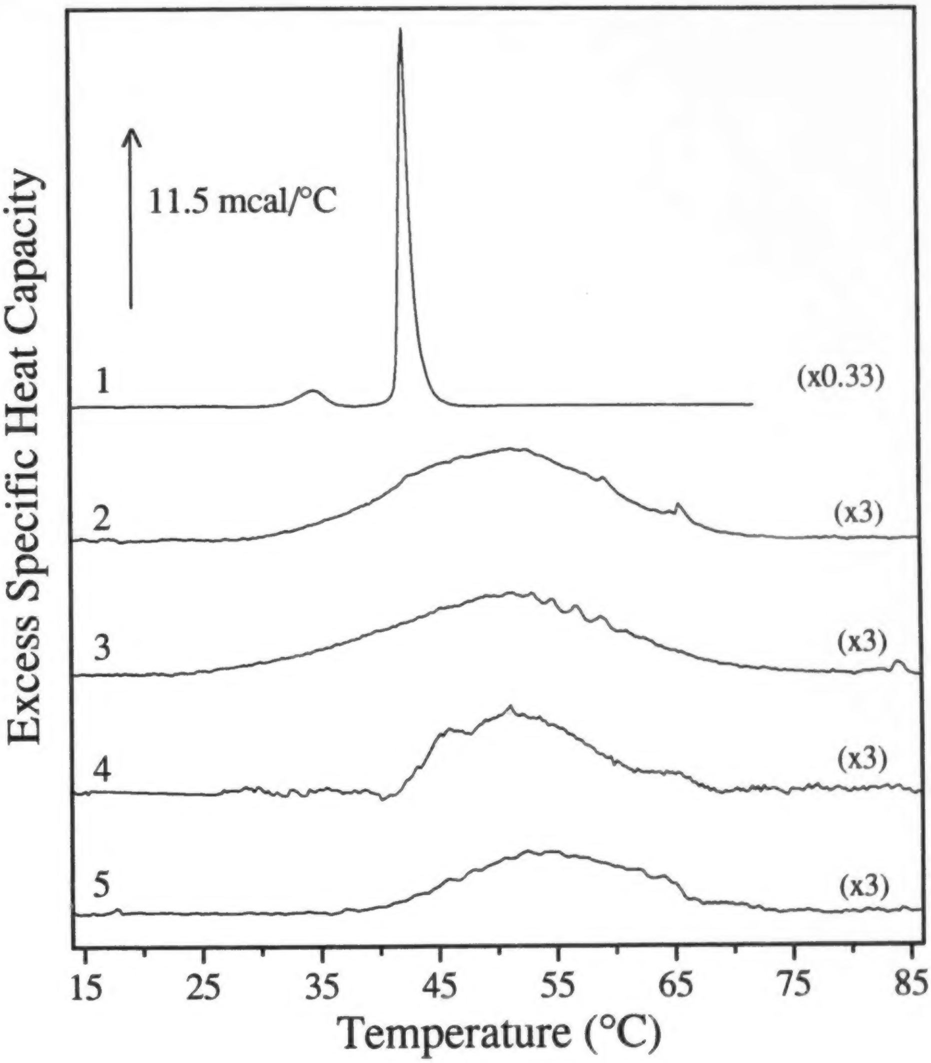

Figure 3.5 DSC thermograms of DPPC liposomes. 1. pure DPPC; 2. DPPC:CH:DCP (3:1:0.5 mole ratio); 3. DPPC:CH:DCP containing AraC; 4. OPP-coated DPPC:CH:DCP; 5. OPP-coated DPPC:CH:DCP liposomes containing AraC.

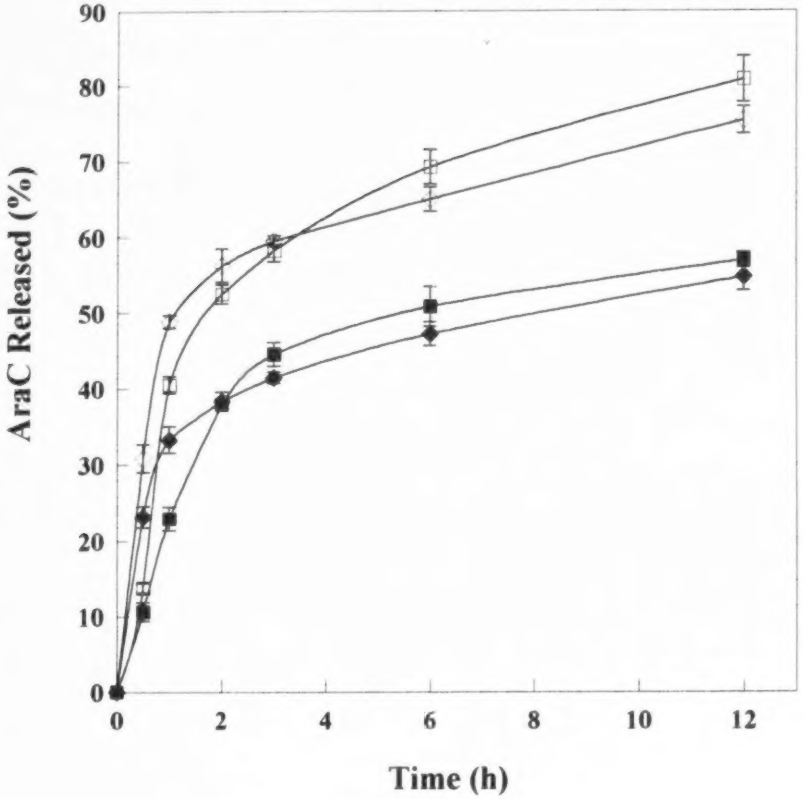

Figure 3.6 Release kinetics of AraC from uncoated (open symbols) and OPP-coated (closed symbols) liposomes in simulated gastric fluid USP, pH 1.2, 37°C. ◇, ◆, DMPC:CH:DCP (3:1:2 mole ratio); □, ■, DPPC:CH:DCP (3:1:0.5 mole ratio); Means±SEM, n=3.

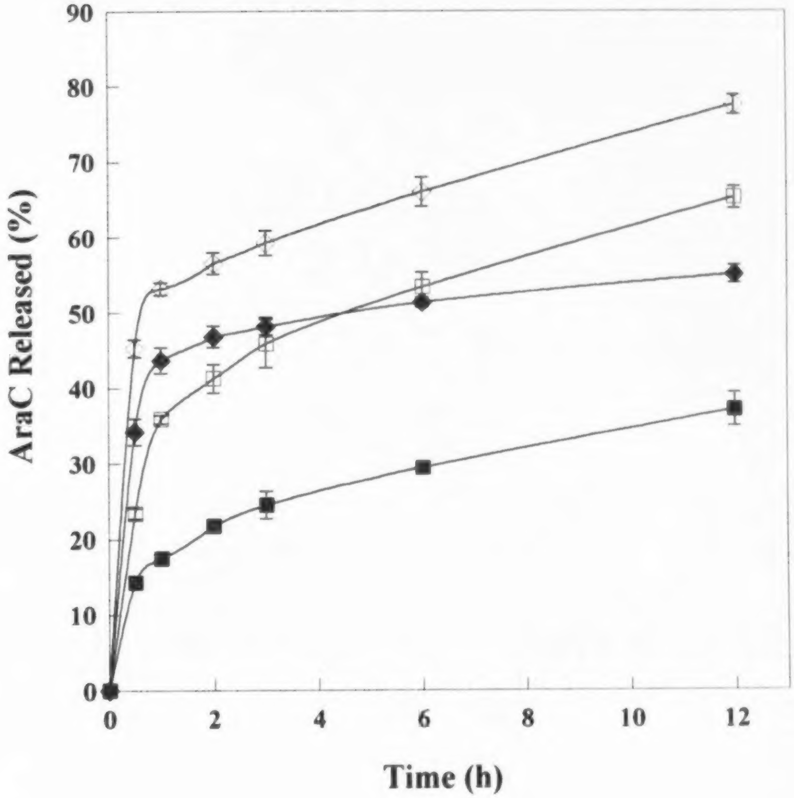

Figure 3.7 Release kinetics of AraC from uncoated (open symbols) and OPP-coated (closed symbols) liposomes in simulated intestinal fluid USP, pH 7.4, 37°C. ◇, ◆, DMPC:CH:DCP (3:1:2 mole ratio); □, ■, DPPC:CH:DCP (3:1:0.5 mole ratio); Means±SEM, n=3.

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Chapter 4

CHARACTERIZATION OF CHOLESTEROL PULLULAN-COATED LIPOSOMES INTENDED FOR ORAL ADMINISTRATION

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4.1. Introduction

Liposomes have been investigated extensively as potential drug carriers. A detailed characterization of the structure of polymer-coated liposomes, including particle size distribution, coating efficiency and the effect of polymer on encapsulation efficiency of entrapped solutes and on particle size, and their release characteristics are often neglected. A detailed characterization is important in order to interpret the differences in release characteristics of entrapped solutes caused by the changes in the physical state of phospholipids and the effect of coating by polymers. Differences in the physical state of phospholipids, *i.e.*, liquid-crystalline vs. gel-state, affect the physical stability and permeability of the resultant liposomes.

In order to mimic the morphology and function of the cell walls of plant and bacterial cell membranes, several water-soluble and naturally-occuring polysaccharides have been hydrophobized by fatty acids and then adsorbed on cell surfaces. For example, it was first demonstarted that a derivatized polysaccharides easily adsorb onto erythrocyte surfaces (*Tsumita and Ohashi, 1964*). Thereafter, it was observed that such acylated polysaccharides adhere to the surfaces of cytoplasmic membranes (*Hammerling and Westphal, 1967*), planar lipid bilayer membranes (*Wolf et al., 1977*) and liposomes (*Sunamoto et al., 1983; Kobayashi et al., 1986*). Sunamoto and coworkers have added a palmitoyl group as a hydrophobic anchor to several naturally-occuring polysaccharides (*Sunamoto et al., 1983; Sunamoto and Iwamoto, 1985*) including mannan, levan, amylopectin, dextran, amylose and pullulan. Improved stability of liposomes coated with palmitoyl or cholesterol-substituted polysaccharides was also reported with respect to decreased permeability of a water-soluble probe, 6-carboxyfluorescein, which was entrapped in the interior aqueous compartments of liposomes. The liposomes exhibited increased resistance against enzymatic lysis with phospholipase-D and lipid peroxidation (*Sunamoto et al., 1987*). Polysaccharide-coated liposomes have also been employed as receptor-mediated drug carriers (*Takada et al., 1984*) due to their decreased immunogenicity and better biocompatibility.

Surfactants and lysolecithins, which bear single long alkyl chains, are known to make the lipid membranes more leaky and often destroy the integrity of lipid bilayer membranes (*Anzai et al., 1980; Kunimoto et al., 1981; Nakazawa et al., 1984*). On the other hand, the addition of cholesterol into the lipid membrane makes the membrane more stable against hostile environmental influences (*de Gier et al., 1968; Seelig and Seelig, 1974*). Thus pullulan was derivatized with cholesterol molecules to anchor it on liposome surfaces as attempted previously (*Sunamoto et al., 1992*). The aim of the work reported here was to improve the physical stability of liposomes by coating with cholesterolpullulan (CHP), and thereafter, to compare the permeability of uncoated and CHP-coated liposomes in simulated gastric and intestinal fluids in order to evaluate their potential as a oral liposome formulation.

4.2. Materials and Methods

4.2.1. Materials

L-α-dimyristoylphosphatidylcholine(>99%),L-α-dipalmitoylphosphatidylcholine (>99%), dicetylphosphate (DCP), cholesterol (CH, >99%), pullulan (average mol. wt. 54,000D), ethylenediamine, 1-ethyl-3-(3-dimethylamino)propylcarbodiimide, and cytosine-ß-D-arabinofuranoside (AraC) were used as received from Sigma Chemical Co., St. Louis, U.S.A. Potassium dihydrogen phosphate (PDP), sodium hydroxide, dimethylformamide and isopropanol were obtained from BDH Inc., Toronto, Canada. Water was demineralized and twice-distilled before use.

4.2.2. Synthesis of CholesterolPullulan

Cholesterol was combined with pullulan *via* a aminoethylcarbamoylmethyl (AECM) spacer according to the procedure described (*Sunamoto et al., 1987*).

Carboxymethylated pullulan was prepared by reacting 3 g of pullulan with 3.2 g of sodium monochloroacetate in 55 ml of 1 M NaOH. Fifteen millilitres of 10 M NaOH solution was added, and the mixture diluted to 100 ml with distilled water. The reaction mixture was stirred at 45°C for 12 h, then the pH was adjusted to 7 with 5 ml of 1 M NaH_2PO_4, then 5 M of HCl. This system was dialyzed against water for 4 days, then distilled water for 1 day, and finally condensed to 50 ml under reduced pressure.

The coupling agent 1-ethyl-3-(3-dimethylamino)propyl-carbodiimide (0.65 g) was added followed by 2.1 g of ethylenediamine and, the pH was adjusted to 4.7. The reaction was carried out at 45°C for 12 h with constant stirring. The AECM-pullulan was dialyzed against 0.2 M NaOH solution for 4 days and distilled water for 1 day, then recovered from solution by lyophilization. Cholesterol was introduced by adding 2.73 mg of cholesteryl chloroformate in 12 ml of dry DMF to AECM-pullulan in 25 ml of dry DMSO. The reaction was carried out at 85°C for at least 15 h in the presence of 5 ml of dry pyridine, then it was added to 100 ml of ethanol and a white precipitate was obtained. The product was washed with ethanol and diethylether to remove free cholesterol and other impurities, dissolved in water and then lyophilized. The degree of cholesterol substitution was determined by ^1H-NMR analysis of CHP dissolved in d_6-DMSO at room temperature (23 ± 2°C).

4.2.3. Preparation of Liposomes

Solution of lipids in chloroform were evaporated (rotary evaporator) to form thin lipid film on the walls of round-bottom flasks. The flasks were flushed with N_2 gas, then dried under vacuum overnight at 40°C. Subsequently, the lipid film was hydrated with a solution of 30 mM AraC in 5 mM, pH 4.5 PDP solution yielding a final lipid concentration of 15 mM and a lipid:drug mole ratio of 1:2. Dispersion and swelling of the lipid were carried out above the phase transition temperatures (T_m) of the respective phospholipids. The multilamellar vesicles (MLVs) were vortex-mixed, then subjected to five freeze-thaw (FT) cycles forming FATMLVs.

Polysaccharide-coated liposomes were prepared by adding 1.5 ml of a 0.5 percent CHP solution in PDP, to 3 ml of liposomal suspension followed by intermittent stirring for a minimum of 3 h, then stored at 4°C. Uncoated liposomes were similarly treated but without the polymer. The liposomes were centrifuged (Beckman Model L8-55 ultracentrifuge, 150,000 x g, 15°C, 30 min) and the supernatant containing untrapped drug and polymer were removed.

4.2.4. Determination of CHP Coating Efficiency

The amount of CHP which coated liposomes was determined by a method reported previously (*Dong and Rogers, 1991*). Briefly, 1 ml of the CHP-coated liposomes were ultracentrifuged and the pellet was diluted to 10 ml with 1:1 v/v isopropanol:PDP solution. After 15 min, absorbances were recorded at 400 nm and the CHP concentration determined from a calibration curve. Absorbances were corrected for small lipid absorbances by similarly treating uncoated liposomes. CE was calculated as a ratio of the weight (mg) of polymer in CHP-coated liposomes to the weight (mg) of lipids in the liposome suspension.

4.2.5. Determination of Encapsulation Efficiency

Uncoated and CHP-coated liposomes (2 ml) were ultracentrifuged, then the pellet was dissolved in 100 ml of isopropanol:PDP (25:75 v/v) solution. AraC was analyzed by reverse-phase HPLC at 273 nm (Novapak C_{18}, 15-cm column; mobile phase of 2 percent methanol in pH 4.5 PDP solution, at a pump rate of 1 ml/min) (*Kim and Howell, 1987*). Concentrations of AraC were determined from calibration curves (0-30 μg/ml) in either PDP solution only or in 25:75 v/v isopropanol:PDP system ($r^2=1.00$, $n=12$ in either case). EE was expressed as the ratio of the amount of AraC in the pellet to the initial amount of AraC added during liposome preparation.

4.2.6. Sedimentation, Microscopy and Particle Sizing Studies

Uncoated and CHP-coated DMPC:CH:DCP and DPPC:CH:DCP composition liposomes were allowed to sediment for 175 h under the effect of gravity and then were centrifuged in hemocrit tubes and the sedimentation volumes (V_{sed}) were recorded. Liposomes were observed under an optical microscope (Olympus 219738, x1000 magnification), and the freeze-fractured liposomes were studied by transmission electron microscopy (TEM, Hitachi H-7000).

Particle size distributions of uncoated and CHP-coated liposomes were determined by laser light scattering (Brookhaven Instruments BI-90 Particle Sizer) of a 100 μl sample diluted to 3 ml with PDP solution. The dust factor was maintained at 0.01.

4.2.7. Differential Scanning Calorimetry Studies

A high-sensitivity differential scanning calorimeter (Hart Scientific DSC, Model 4207) was employed to evaluate the relative thermotropic phase behavior of liposomes.

Phospholipid dispersions (20 mg/ml) of DPPC and DPPC:CH:DCP (3:1:0.5 mole ratio) composition with and without AraC or CHP were analyzed. Samples were scanned between 10 and 90°C at a rate of 15°C/hour for both heating and cooling scan modes. At least three scans were carried out to ensure equilibrium and reproducibility. Endothermic enthalpies, T_m and peak half-widths ($\delta T_{1/2}$) were recorded and analyzed by Origin software (Microcal).

4.2.8. Determination of Release Kinetics in Simulated GI Fluids

The liposome pellets were suspended to 25 ml in simulated gastric (SG) and simulated intestinal (SI) fluids at 37°C. Adjustments were made to ensure that equal concentrations of AraC were present in uncoated and CHP-coated liposomes of either composition. The liposome suspensions were then incubated at 37°C in a Dubnoff Metabolic Shaker. Samples of 250 μl were removed at various time intervals for 12 h, centrifuged (14,000 rpm, 23°C, 4 min) (Eppendorf Centrifuge 5415) and the amount of AraC released in the supernatant was analyzed by HPLC. The fraction of AraC released from liposomes, under sink conditions, was determined as,

$$\%\ \text{Released} = (C-C_o)/(C_T-C_o) \times 100$$

where C_o is the initial drug concentration in the supernatant before incubation, C_T is the total drug concentration in the liposomes, and C is the concentration in the supernatant at time, t (*Dong and Rogers, 1991*). Statistical comparisons between the fraction of AraC released from uncoated and CHP-coated liposomes were made using one-way ANOVA ($\alpha = 0.05$).

4.3. Results and Discussion

The degree of substitution of pullulan by cholesterol as the hydrophobic anchor was 0.5 percent or one CH molecule per 200 saccharide units (*i.e.*, each pullulan monomer) based on the conditions employed. This provided a product which had suitable solubility behavior to be used to coat liposomes. The ^{1}H-NMR spectrum of CHP yielded signals between 0.6 ppm and 1.4 ppm corresponding to the methyl and methylene protons of the CH molecule. In contrast, these signals were absent in the ^{1}H-NMR of pullulan. This compares to the results reported previously (*Sunamoto et al., 1987*).

4.3.1. Coating Efficiency of Liposomes by CHP

Coating liposomes with a polymer is dependent on the degree of interaction between the polymer and the liposome surfaces. Many polymers such as pullulan, dextran or carboxylic acids are highly water-soluble and do not spontaneously coat dynamic liposome systems to produce stable films. Instead, a hydrophobic moiety is required to anchor the polymer chains to the bilayer surfaces (*Sunamoto and Iwamoto, 1985*).

The CE of liposomes as a function of CHP concentration is shown in Figure 4.1. The CE for DMPC:CH:DCP (3:1:2) and DPPC:CH:DCP (3:1:0.5) liposomes reached a maximum at an initial CHP concentration of 0.5 percent which compares with the optimal coating at 0.6 percent concentration of silicone polymer on phospholipid surfaces reported by others (*Khattab et al., 1989*). The CE decreased with an increase of CHP concentration for both liposome compositions, except that the DMPC composition also exhibited another small maximum at 2.5 percent CHP suggesting that the polymer has the potential of penetrating the fluid state bilayers deeper, as expected. Liposomes were coated with 0.5 percent CHP solutions for further studies.

4.3.2. Encapsulation Efficiency and Liposome Size Distribution

The EE of AraC in uncoated and CHP-coated liposomes of DMPC:CH:DCP and DPPC:CH:DCP compositions are shown in Table 4.1. The EEs were higher in DPPC than in DMPC liposome compositions due to the gel-state of DPPC bilayers at 23°C which are better structured, and hence, less leaky. Previous results have shown that phospholipid:cholesterol mole ratio of 3:1 appeared to have the most favorable characteristics and maximum EE of 28 and 40 percent were obtained in uncoated DMPC:CH:DCP and DPPC:CH:DCP liposomes, respectively (data obtained from chapter 2). Coating these liposomes with CHP caused some reduction in the EE of AraC which may possibly be related to AraC's displacement from surface bilayer binding sites.

The particle size distributions of uncoated and CHP-coated liposomes of both compositions followed log-normal particle size distribution (Figures 4.2 and 4.3). A signicant increase ($p<0.05$) in the particle mean diameter in the absence of any aggregation are indicative of CHP being associated with the lipid bilayers, and most likely on outer bilayer surfaces.

4.3.3. Evidence of CHP-Bilayers Interaction

Uncoated liposomes appeared as transparent, spherical vesicles when observed under the optical microscope (x1000 magnification). The CHP-coated liposomes, however, were observed as translucent with noticeable wall material surrounding them.

Transmission electron micrographs of freeze-fractured liposomes are shown in Figures 4.4 and 4.5 for the DMPC:CH:DCP and DPPC:CH:DCP compositions, respectively. A clear distinction can be seen between uncoated and CHP-coated liposomes, where the latter appear to have a uniform outer film of polymer adsorbed.

Furthermore, the uncoated liposomes in both cases contained small indentations in all freeze-fractured micrographs indicating phase separation within the bilayers, that were not visible in polymer-coated liposomes.

Sedimentation volume (V_{sed}) of uncoated and CHP-coated liposomes (1 ml each) of either composition were determined following sedimentation under the effect of gravity for 175 h (Figure 4.6), and then, following centrifugation (Figure 4.7). The V_{sed} values of CHP-coated liposome dispersions were higher in both liposome compositions, before and after centrifugation (Figures 4.6 and 4.7), confirming that the CHP adsorbed on liposome bilayer surfaces, producing relatively flocculated liposome dispersions.

4.3.4. Characterization of Liposomes by DSC

The thermotropic behavior of DMPC and DPPC liposomes was qualitatively similar, hence only results for the DPPC composition are reported. The effects of polymer-coating on the physical state of DPPC:CH:DCP (3:1:0.5 mole ratio) bilayers in the presence or absence of AraC are indicated in the DSC thermograms in Figure 4.8. Pure DPPC bilayers exhibited two transitions during heating, a pre-transition temperature at 38°C and the main transition at 41.5°C (curve 1) which is characteristic for this phospholipid (20). The liposome composition of DPPC:CH:DCP gave a main endothermic peak at 51.2°C, due to the increased number of hydrocarbon chains, and a $\delta T_{1/2}$ which was considerably elevated, indicating a lower chain cooperativity in the bilayers (curve 2, Table 4.2). The encapsulation of AraC (40 percent) resulted in a slight reduction in the T_m and $\delta T_{1/2}$ (curve 3 in Figure 4.8, Table 4.2), suggesting that binding of AraC to negatively-charged DCP sites led to increased cooperativity in the bilayers. These effects may be due to deeper penetration of CH molecules in the bilayers as a result of the AraC-DCP binding at the surfaces. Coating DPPC:CH:DCP liposomes with CHP produced a larger quantitative reduction of $\delta T_{1/2}$ or an increase in cooperativity within the bilayers accompanied by a secondary increase in T_m (curve 4, Table 4.2). This result indicates ordering of the bilayers by CHP, presumably due to its surface attachment (Figures 4.2 and 4.3). In other words, the polymer restricts movement of the bilayer molecules in the polar head group region. Coating liposomes containing AraC with CHP caused a slight increase of T_m and a significant increase of $\delta T_{1/2}$ (curve 5, Table 4.2) compared to AraC-free liposomes. These changes are likely connected to some displacement of AraC from bound sites (Table 4.1) coupled with a corresponding reduction of penetration of CH in the bilayers, thereby reducing the restrictive influence of CHP.

4.3.5. Kinetics of Release from Uncoated and CHP-coated Liposomes

The release profiles of AraC from uncoated and CHP-coated DMPC and DPPC liposome compositions in SG and SI fluids are shown in Figures 4.9 and 4.10, respectively. Each is characterized by an initial rapid release phase of AraC from liposomes after resuspending the liposomes followed by a slower rate after about 2 h, which obeyed first-order kinetics and lasted for 12 h. In SG fluid (Figure 4.9), almost 61 percent of encapsulated AraC was released from uncoated DMPC liposomes within 2 h whereas only 38 percent was released from CHP-coated liposomes. Also, the first-order rate constant, k_1, from 2-12 h was significantly ($p < 0.05$) lower from CHP-coated liposomes (Table 4.3). The ratio, k_1^u/k_1^c, corresponding to the 1st-order rate constants of uncoated and CHP-coated liposomes was 2.0 for both the DMPC:CH:DCP and DPPC:CH:DCP liposome compositions. Thus, coating liposomes with CHP reduced the leakage rate of the remaining AraC by 100 percent. Consequently, approximately 50 percent of the AraC still remained encapsulated after 12 h in CHP-coated liposomes in SG fluids at 37°C.

In SI fluid (Figure 4.10), the initial rapid release of AraC in uncoated liposomes of DPPC composition was 15 percent less than that of DMPC composition (Table 4.3). This difference, compared to an insignificant difference in SG fluid, is likely due to the influence of pH on the ionization of AraC, its binding to the negatively-charged DCP molecules, and the fluidity of the bilayers. Exposing the liposomes to pH 7.4 medium, in which AraC is less ionized, should cause less binding to the bilayers resulting in leakage of unionized AraC. Liposomes of DMPC composition exist in the fluid state at 37°C (T_m = 23°C), and generally these have higher permeabilities than liposomes of DPPC composition, which exist in the gel state at that temperature.

Liposomes of DPPC composition coated with CHP released only 20 percent of the entrapped AraC within 2 h compared to 40 percent released from CHP-coated DMPC composition (Table 4.3). Beyond 2 h, the release of AraC proceeded by a 1st-order kinetic process and the ratio, k_1^u/k_1^c, was 1.3 and 1.7 for the DMPC and DPPC composition liposomes, respectively. After 12 h, only 36 percent of AraC was lost from liposomes of DPPC compared to 62 percent from liposomes of DMPC composition.

The encapsulation and release of the small, water-soluble model solute, AraC are determined by the liposome composition and the pH of the release medium. Gel-state liposomes allowed higher EEs and slower release rates than the liquid-crystalline state liposomes, even though some AraC is electrostatically bound to the bilayers. Coating the liposomes with the polysaccharide, CHP, significantly reduced the initial leakage and the rate of release up to 12 h. The main mechanism appears to be due to increased ordering of the outer bilayers due to anchoring of the pullulan at the outer bilayer surfaces. In SG fluid at pH 1.2, uncoated liposomes were particularly leaky but this was reduced substantially by coating with CHP. A similar pattern was followed in SI fluid at pH 7.4 except that the uncoated liposomes were less permeable to AraC than at pH 1.2. Again, coating by CHP reduced the permeability, particularly of gel-state liposomes indicating that the DPPC:CH:DCP liposome formulation may have the potential to deliver intact solute to the lower regions of the GI tract following oral administration.

Acknowledgements

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Table 4.1 **Percent Encapsulation Efficiency of AraC in Uncoated and CHP-coated Liposomes at pH 4.5[a]**

Liposome Composition[b]	Uncoated[c]	CHP-coated
DMPC:CHOL:DCP (3:1:2)	28±2.7	24±1.8
DPPC:CHOL:DCP (3:1:0.5)	40.2±1.4	36.5±1.2

[a]calculated as the fraction of initial AraC concentration (30 mM), Means±SEM, n=6,

[b]numbers in brackets are the mole ratios of the lipid components, the total lipid concentration was 15 mM,

[c]taken from Table 2.1.

Table 4.2 **Endothermic T_m and $\delta T_{1/2}$ values of DPPC:CH:DCP (3:1:0.5 mole ratio) Liposomes**

Curve Number[a]	T_m (°C)	$\delta T_{1/2}$
1	41.5	0.45
2	51.2	26.0
3[b]	50.5	24.5
4	53.1	15.6
5[b]	53.9	20.3

[a] from DSC curves (see Figure 4.7),

[b] liposomes containing AraC.

Table 4.3 **Release of AraC from Uncoated and CHP-coated DMPC:CH:DCP and DPPC:CH:DCP Liposomes in SG and SI fluids USP, 37°C**

Release Medium	Liposome Type	% AraC Released at 2 h	12 h	k_1 (h^{-1})	k_1^u/k_1^c
SG, pH 1.2	Uncoated DMPC[a]	61	86	5.4±1.1	
	CHP-coated DMPC	38	51	2.7±0.3	2.0
	Uncoated DPPC[b]	55	81	5.7±0.3	
	CHP-coated DPPC	31	45	2.8±0.3	2.0
SI, pH 7.4	Uncoated DMPC	59	86	6.6±0.4	
	CHP-coated DMPC	40	62	5.0±0.5	1.3
	Uncoated DPPC	44	70	6.0±0.4	
	CHP-coated DPPC	20	36	3.6±0.4	1.7

[a] DMPC:CH:DCP (3:1:2 mole ratio) liposomes,

[b] DPPC:CH:DCP (3:1:0.5 mole ratio) liposomes,

k_1^u and k_1^c were derived from the slopes of the 2-12 h release phase from uncoated and CHP-coated liposomes, respectively,

Means±SEM, n=3.

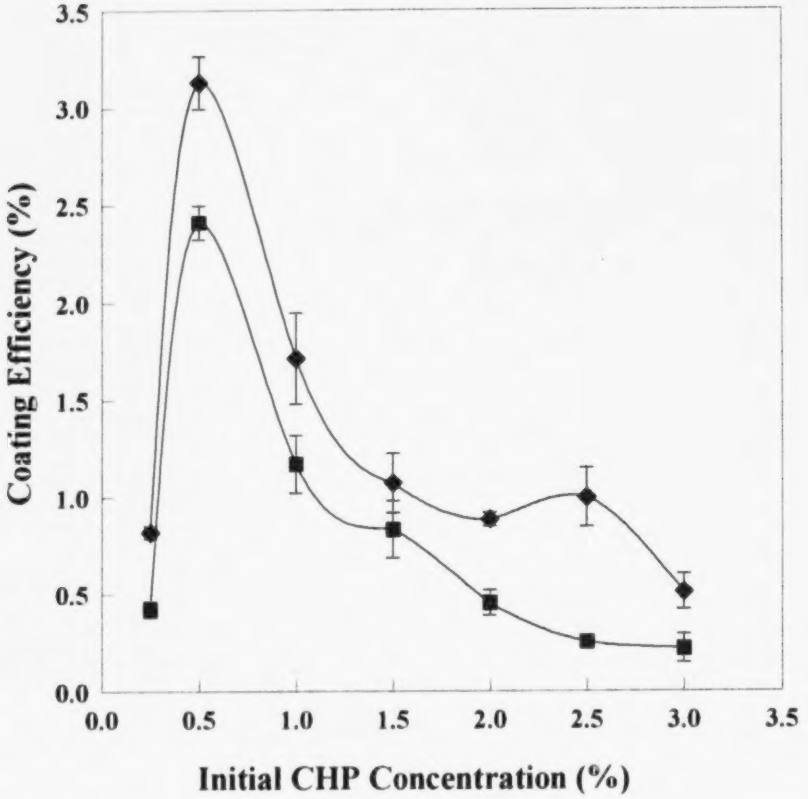

Figure 4.1 Coating efficiency (%) of liposomes by CHP as a function of the initial CHP concentration at pH 4.5. ◆, DMPC:CHOL:DCP (3:1:2 mole ratio); ■, DPPC:CHOL:DCP (3:1:0.5 mole ratio); Means±SEM, n=4.

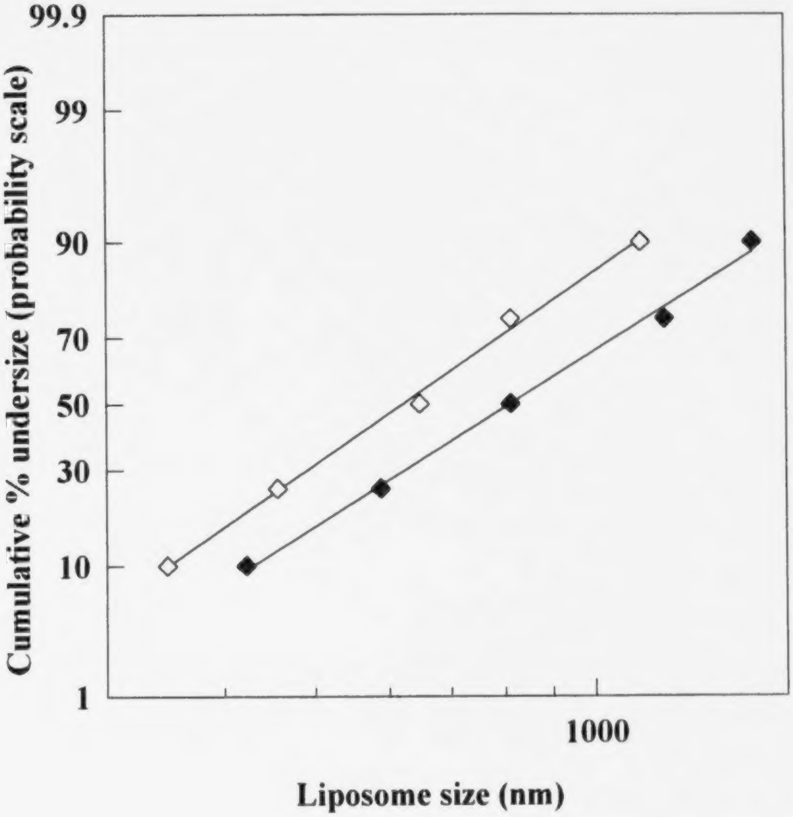

Figure 4.2 Log-probability plots of size of DMPC:CH:DCP (3:1:2 mole ratio) uncoated (open symbols) and CHP-coated (closed symbols) liposomes, n=3.

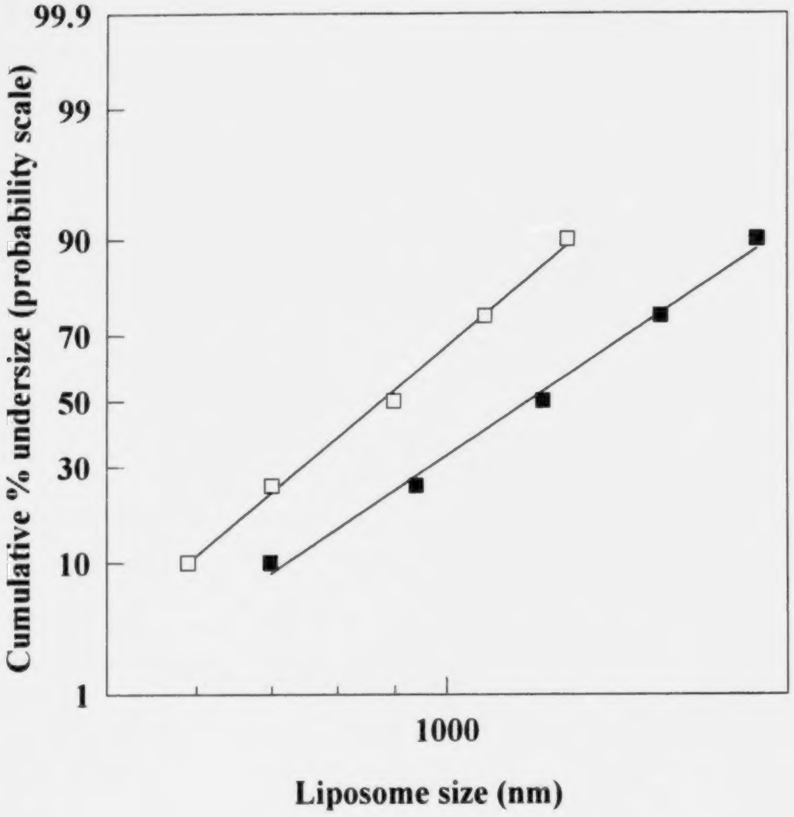

Figure 4.3 Log-probability plots of size of DPPC:CH:DCP (3:1:0.5 mole ratio) uncoated (open symbols) and CHP-coated (closed symbols) liposomes, n=3.

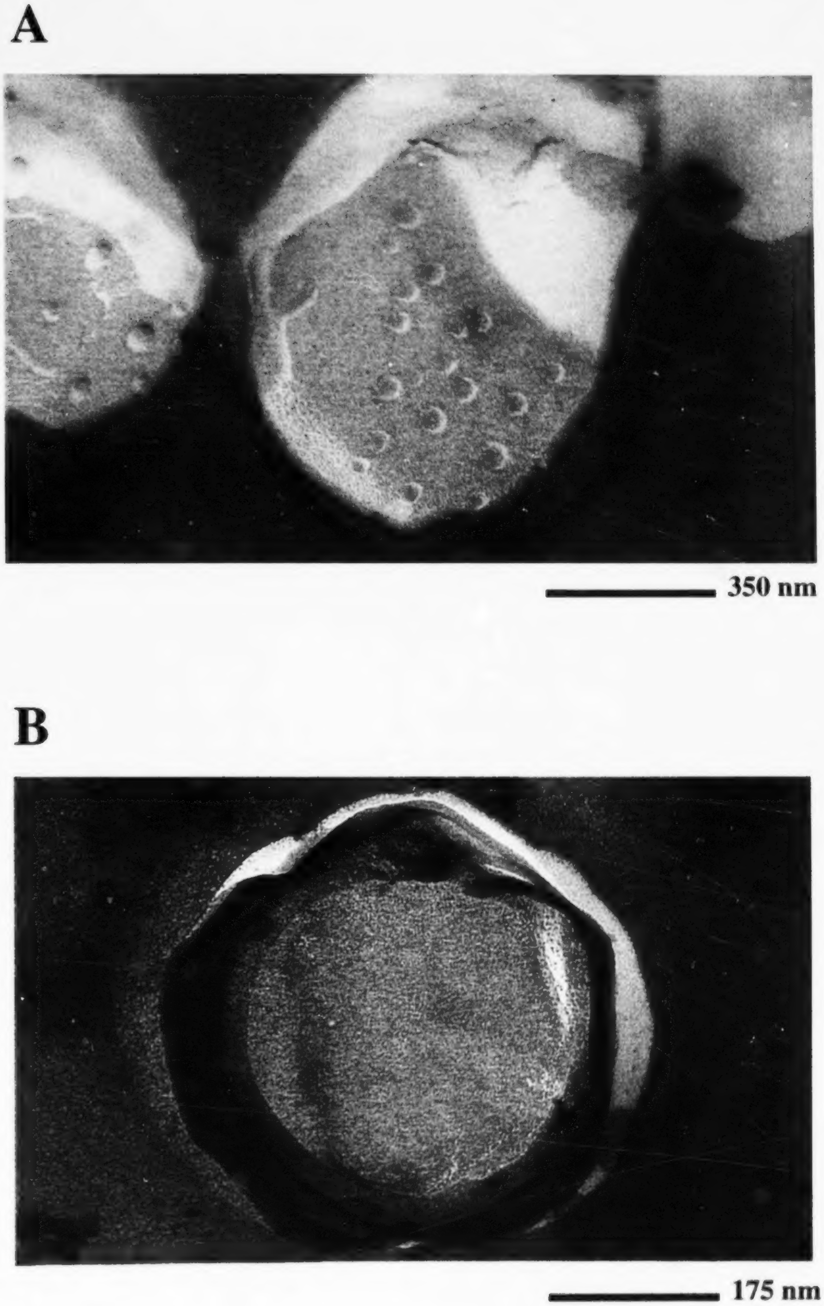
A
350 nm
B
175 nm

Figure 4.4. Transmission electron micrographs of DMPC:CH:DCP (3:1:2 mole ratio) freeze-fractured liposomes.(**A**.) Uncoated;(**B**.) CHP-coated.

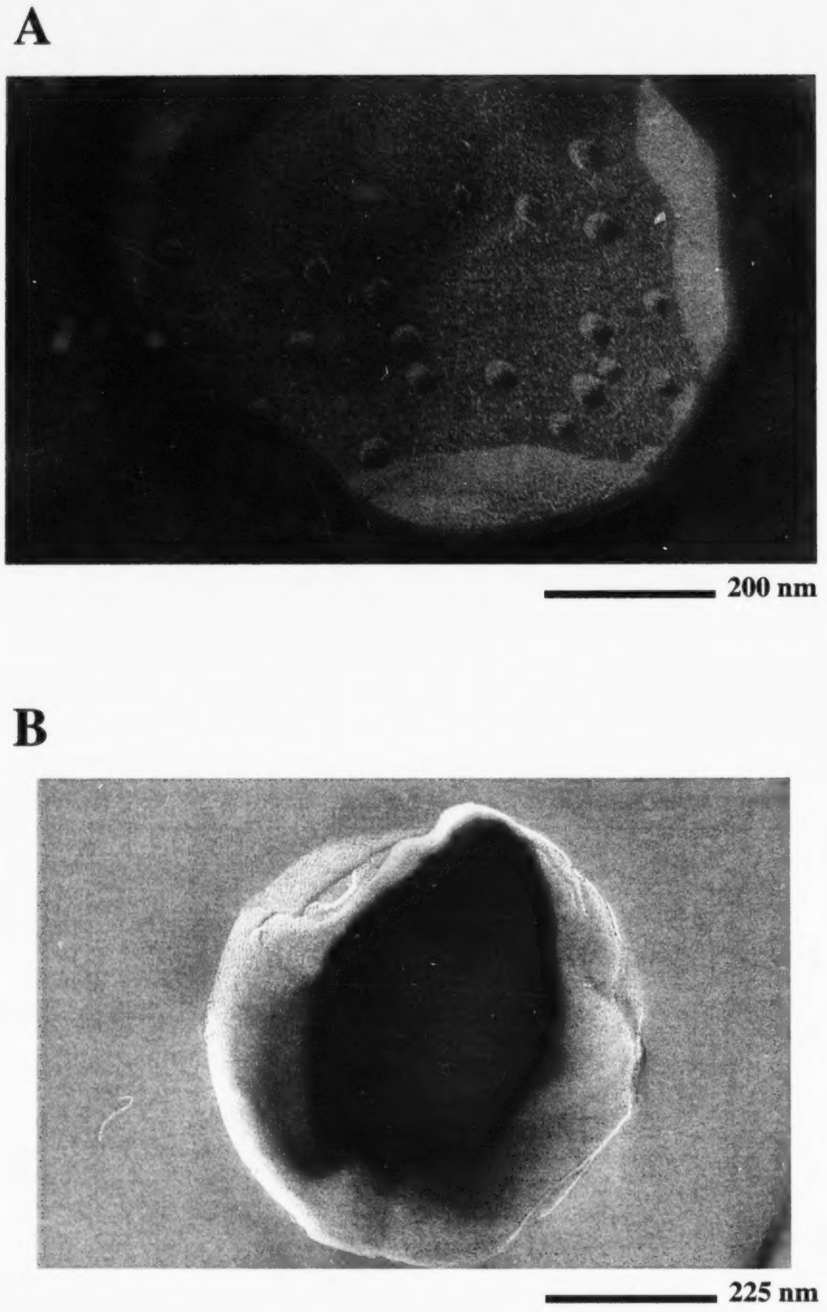

Figure 4.5. Transmission electron micrographs of DPPC:CH:DCP (3:1:0.5 mole ratio) freeze-fractured liposomes. (**A**.) Uncoated;(**B**.) CHP-coated.

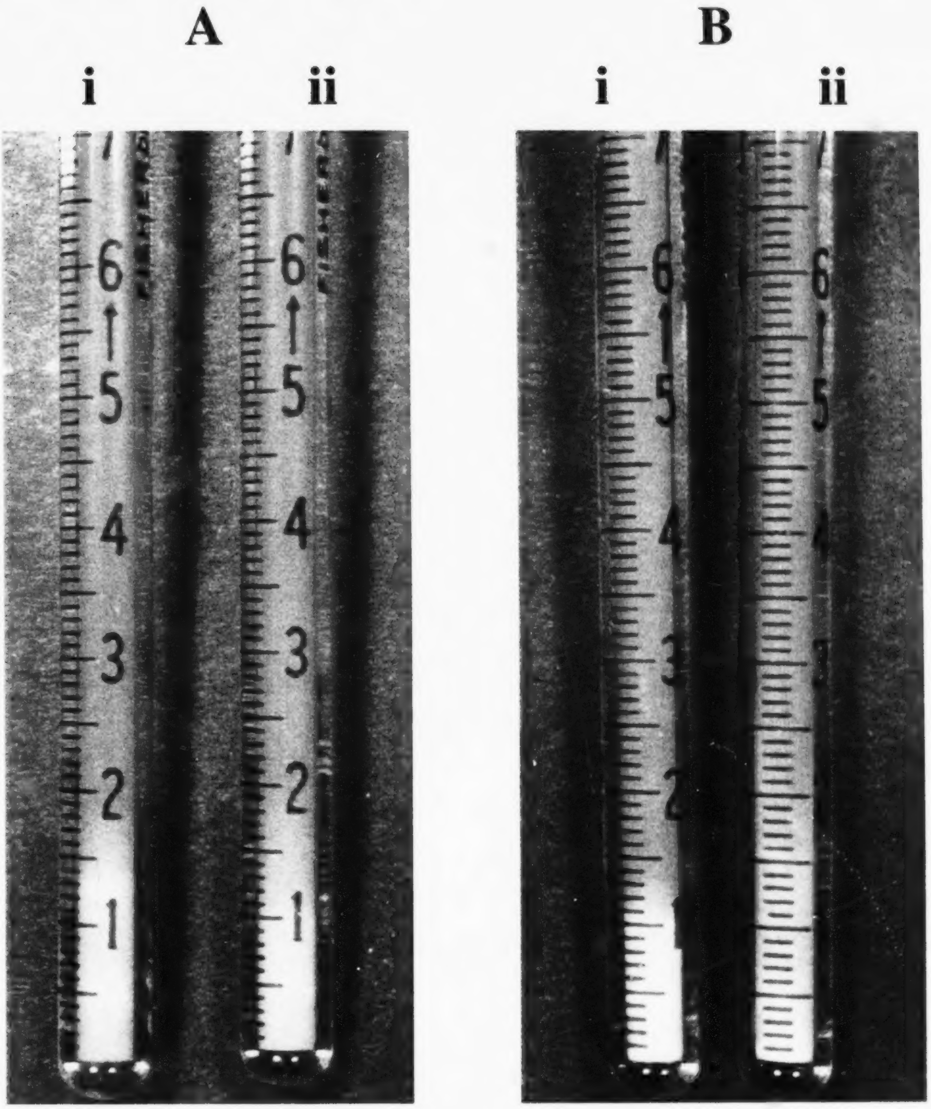
A
i
ii
B
i
ii
7
6
5
4
3
2
1
FISHER

Figure 4.6. Sedimentation of **(i)** uncoated and **(ii)** CHP-coated liposomes after 175 hours in 1 ml Wintrobe blood sedimentation tubes. **(A)** DPPC:CH:DCP (3:1:0.5 mole ratio); **(B)** DMPC:CH:DCP (3:1:2 mole ratio).

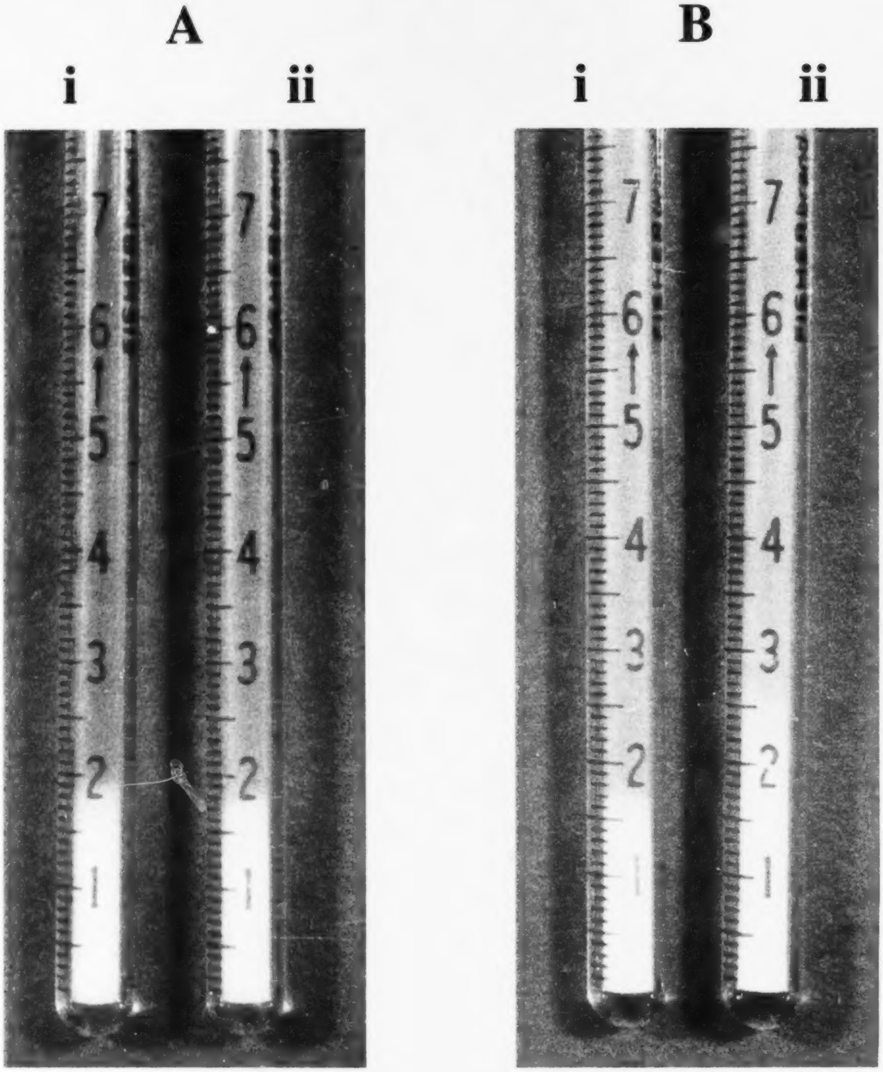

Figure 4.7. Sedimentation of **(i)** uncoated and **(ii)** CHP-coated liposomes after 175 hours under normal gravity, then 30 minutes at 1000 g. **(A)** DPPC:CH:DCP (3:1:0.5 mole ratio); **(B)** DMPC:CH:DCP (3:1:2 mole ratio).

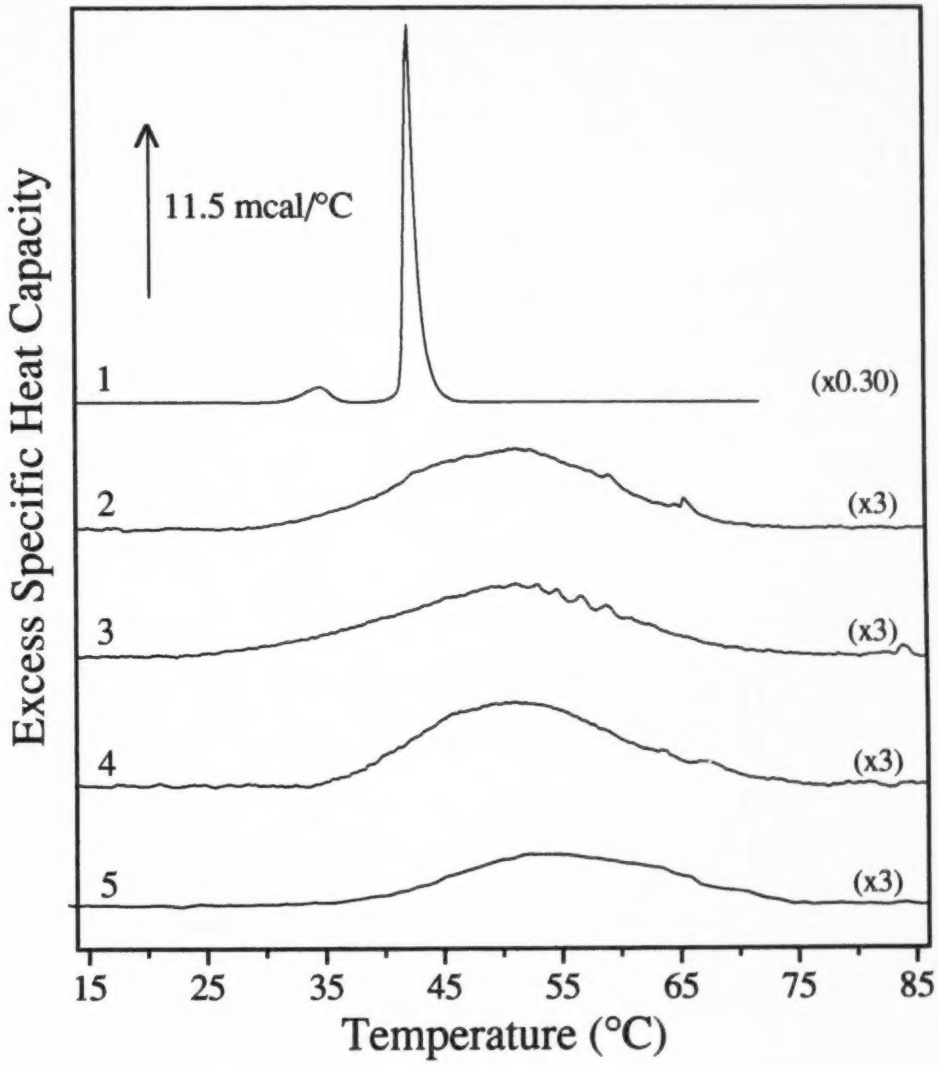

Figure 4.8 DSC thermograms of DPPC liposome compositions, **1**. pure DPPC; **2**. DPPC:CH:DCP (3:1:0.5 mole ratio); **3**. DPPC:CH:DCP containing AraC; **4**. CHP-coated DPPC:CH:DCP; **5**. CHP-coated DPPC:CH: DCP containing AraC.

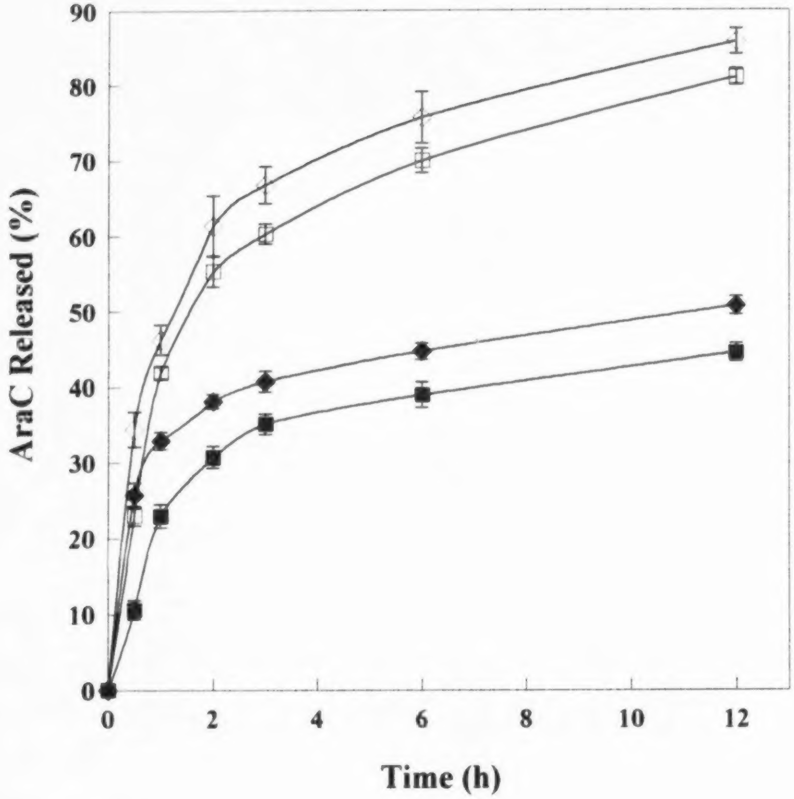

Figure 4.9 Release kinetics of AraC from uncoated (open symbols) and CHP-coated (closed symbols) liposomes in simulated gastric fluid USP, pH 1.2, 37ºC. ◇, ◆, DMPC:CHOL:DCP (3:1:2 mole ratio); □, ■, DPPC:CHOL:DCP (3:1:0.5 mole ratio); Means±SEM, n=3.

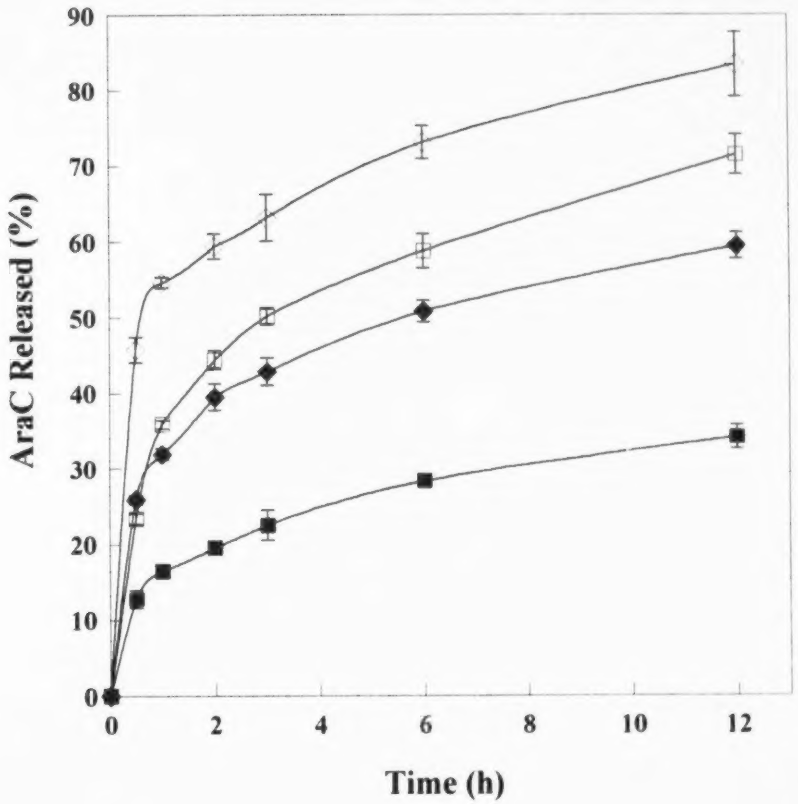

Figure 4.10 Release kinetics of AraC from uncoated (open symbols) and CHP-coated (closed symbols) liposomes in simulated intestinal fluid USP, pH 7.4, 37°C. ◇, ◆, DMPC:CHOL:DCP (3:1:2 mole ratio); □, ■, DPPC:CHOL:DCP (3:1:0.5 mole ratio); Means±SEM, n=3.

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Chapter 5

FORMULATION AND *IN VITRO* RELEASE OF A MODEL PEPTIDE FROM PULLULAN-COATED LIPOSOMES

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5.1. Introduction

Advances in cell and molecular biology have led to a greater awareness of the therapeutic values of peptides and proteins. At present, almost all therapeutic peptide and protein drugs are administered parenterally and this has been associated with poor patient compliance. The successful delivery of peptides in therapeutically significant quantities by a route other than parenteral is a major challenge.

The physical and chemical properties of proteins do not make them good candidates for oral administration. Their molecular size, often complex secondary, tertiary, and even quaternary structures, and their surface characteristics are properties which are inappropriate for absorption by cell membranes. In order for a drug to be administered orally, it must be able to withstand chemical and enzymatic degradation influences during transit in the lumen of the GI tract and with in the mucosal barrier, as well as metabolism during transepithelial transport and hepatic first-pass transport. Considerable progress has been made in developing oral delivery systems, particularly for small (3-12 amino acid residues) or medium-size (13-60 amino acid residues) peptides. It has been known for some time that some peptides and proteins can cross the walls of the GI tract, albeit in low amounts resulting in poor bioavailabilities (Ma *et al.*, 1993). These include interferon-α, calcitonin, chymotrypsin, horseradish peroxidase, elastase, *Clostridium botulinum* type A toxin, a variety of food antigens, insulin, heparin, and Factor VIII. Recently, the peptide DDAVP (desmopressin, 0.1 and 0.2 mg) was introduced (Ferring Pharmaceuticals, Inc.) as a tablet dosage form representing the first peptide to be marketed for oral administration as an alternative to intranasal DDAVP.

Minimal quantities of orally-administered macromolecules/proteins are absorbed by the GI tract *via* non-specific and specific pathways, but the amounts transported are usually insufficient to achieve therapeutic effects. Recent studies have focussed on increasing their low bioavailabilities rather than exploiting *in vivo* biochemical transport processes. Considerations only of protecting peptides from proteolytic degradation are insufficient to achieve increased bioavailabiltiy because barrier functions of the epithelial membrane and its associated mucus layers also need to be taken into account. The co-administration of protease inhibitors, such as apoprotinin, have resulted in improved absorption behavior of peptides since they perturb the epithelial membrane (transiently) and macromolecules in close proximity cross into the vascular compartment and become bioavailable (*Lee and Yamamoto, 1990; Lee et al., 1991*).

Polymeric microencapsulation of peptides offers several potential advantages in administration and therapy. For example, drugs can be delivered in a sustained or controlled manner at specific sites of absorption. On this basis alone, the problem of instability may be overcome, particularly if the drug can be released in regions that are less hostile, such as the proximal colon. Encapsulated peptides can be protected against degrading enzymes, such as pepsin and peptidases in the GI tract (*Kararli, 1989*), until the solutes are actually released. Coating the particulate carriers by derivatized polysaccharides has been an effective means of reducing their permeabilities in the presence of bile salts (*Walde et al., 1987; Nagata et al., 1990*) and pancreatic phospholipases (*Sunamoto et al., 1983*).

Liposomes have been considered as delivery systems as well as immunoadjuvants for the oral administration of a variety of antigens. They not only allow the inclusion but also are able to protect entrapped antigens from the GI tract conditions and are only weakly nontoxic and immunogenic. Their immunoadjuvant property is belived to be due to the absorption of entrapped antigens *via* Peyer's patches. Functionally, the M-cells on Peyer's patches have been shown to transport macromolecules, particles, and micro-organisms from the GI tract to the underlying lymphoid tissue (*Gilligan and Wan Po, 1991*). Wachsmann *et al.* (*1985*) observed the presence of antigens in the Peyer's patches after the oral administration of liposome-entrapped antigens but not after administration of the soluble antigens alone. *Childers et al.* (*1990*) have shown by transmission electron microscopy that particulate carriers, such as liposomes, are endocytosed by M-cells of Peyer's patches. In a review on the absorption of drugs entrapped within liposomes (*Kimura, 1988*), it was concluded that a small but significant quantity of liposomes were taken up intact by the mucosa of the small intestine, probably by endocytosis.

BP1-16 is a model peptide with 23 amino acid residues (mol. wt. 2,455 Da) and a pI of 7.1 (*Gendler et al., 1990 ; Ding et al., 1993*). It was selected as a model solute to be entrapped in liposomes for peroral delivery *via* the Peyer's patches located mainly in the ileum and jejunum near the ileo-cecal junction. It is a linear chimeric molecule containing two tandom repeats of a single MUC1 epitope plus a tetanus toxin promiscuous T_H epitope (BP1-16 = SAPDTRPASAPDTRPAYSYFPSV; elemental composition $C_{104}H_{153}N_{28}O_{35}$). The permeability of liposomes containing ^{125}I-BP1-16 exposed to environments simulating *in vivo* conditions following oral administration was studied.

5.2. Materials and Methods

5.2.1. Materials

L-α-dioleylphosphatidylcholine (DOPC, >99%), L-α-dipalmitoylphosphatidyl choline (DPPC, >99%), dimyristoylphosphatidylglycerol (DMPG, >99%), cholesterol (CH), pullulan, and palmitoyl chloride were used as received from Sigma Chemicals Co., St. Louis, USA. All other buffer reagents were obtained from BDH Chemicals, Toronto. Water was demineralized and twice-distilled. The peptide BP1-16 and its radioiodinated analogue ^{125}I-BP1-16 were gifts from Biomira Inc., Edmonton, Alberta, Canada.

5.2.2. Synthesis of *O*-PalmitoylPullulan

Two grams of pullulan (average mol. wt. 54,000 da) were dried *in vacuo* at 40°C for at least 3 h. The pullulan was then dissolved in 160 ml of anhydrous formamide and the contents stirred at 60°C in a water-bath for 2 h. Subsequently, 20 ml of anhydrous triethanolamine and 1 g of palmitoyl chloride were added and the contents stirred in a water bath at 60°C for 16 h. The reaction mixture was further diluted with 20 ml of water, then poured into 400 ml of methanol. After standing for five hours at 4°C, the precipitate was collected by filtration, washed with alcohol and dissolved in water. The translucent solution was treated by gel permeation chromatography (Sephadex G-50), the fractions of derivatized polymer were pooled, and then lyophilized (Labconco freeze dryer) (*Hammerling and Westphal 1967*). The degree of substitution of the polysaccharide was estimated from ^{1}H-NMR analysis of a solution of the product dissolved in DMSO-d_6 at room temperature.

5.2.3. Synthesis of CholesterolPullulan

The synthesis of CHP was carried out according to the method described earlier.

(*Sato, 1990*). In the first step, carboxymethylated-pullulan was obtained by reacting 3 g of pullulan with 3.2 g of sodium monochloroacetate in 55 ml of 1 M NaOH. Fifteen milllitres of 10 M NaOH solution was added, then the mixture was diluted to 100 ml with water. The reaction mixture was stirred at 45°C for 12 h, followed by pH adjustment to 7 by adding 5 ml of 1M NaH_2PO_4 followed by 5 M HCl. Thereafter, the mixture was dialyzed against toluene-saturated water for 4 days, then distilled water for 1 day, and finally evaporated to 50 ml under reduced pressure.

The next step involved adding 1-ethyl-3-(3-dimethylamino)propylcarbodiimide (AECM, 0.65 g) as a coupling reagent, followed by 2.1 g of ethylenediamine and the pH was adjusted to 4.7. This reaction was carried out at 45°C for 12 h with constant stirring. The resulting reaction mixture containing AECM-pullulan was dialyzed against 0.2 M NaOH solution for 4 days, then distilled water for 1 day. The product, at this stage, was recovered by lyophilization.

The CH moiety was introduced by adding 12 ml of a solution containing 2.73 mg of cholesteryl chloroformate in dry DMF to a solution of AECM-pullulan in 25 ml of dry DMSO solution. Five millilitres of dry pyridine were added, then the mixture was held at 85°C for at least 15 h. Following this, the reaction mixture was added to 100 ml of ethanol to obtain a white precipitate. This precipitate was washed with ethanol, then diethylether to remove free cholesterol, and any residual reactants, then dissolved in water and lyophilized. The degree of cholesterol substitution in the final product was determined by ^{1}H-NMR analysis in d_6-DMSO.

5.2.4. RadioIodination of BP1-16

Borosilicate glass tubes were coated with iodogen (5 μg per tube in chloroform). Then, BP1-16 was radioiodinated by adding ^{125}I-Na (0.5 μCi) to 100 μg of BP1-16. The radiolabelled peptide was eluted from a Sep-Pak column (C_{18}), previously equilibrated with 0.1 percent trifluoroacetic acid (TFA), by passing 0.1% TFA:AcN (70:30) system. The specific activity of the^{125}I-BP1-16 obtained was $1.83\text{x}10^6$ dpm/μg, and approximately 70 percent of the peptide was labelled. Only the radiolabelled peptide fractions were used in further studies.

The stability of the iodide label on the BP1-16 molecule was confirmed by adding a known quantity of ^{125}I-BP1-16 on a Sep-pak (C_{18}) column, and eluting the column with 0.1% TFA, with which no activity eluted. However, when the elution solvent system was changed to 0.1 % TFA:AcN (70:30), all the activity present on the column eluted.

5.2.5. Preparation of Liposomes

Solutions of the lipids in chloroform were dried as a film in round-bottom flasks using a rotary evaporator. The flasks were flushed with N_2, then dried overnight in a vacuum desiccator at 40°C. Subsequently, the lipid films were hydrated with a solution of the radiolabelled peptide in pH 5.4 acetate buffer (0.17M), yielding a final lipid concentration of 15 mM. Dispersion and swelling of the lipid were carried out at about 12°C above the phase transition temperatures (T_m) of the respective phospholipids. The dispersion was vortex-mixed for 5 min to form multilamellar vesicles (MLVs), then subjected to five freeze-thaw (FT) cycles forming FATMLVs.

Polymer-coated liposomes were prepared by adding 1.5 ml solution (0.5 percent) of OPP or CHP to 3 ml of liposome suspension, stored at R.T. for a minimum of 3 h, then in the refrigerator at 4°C overnight. Uncoated liposomes were similarly treated but without the polymer. The untrapped peptide and unreacted polymer were removed from the supernatant of the liposomal suspension after ultracentrifugation (150,000xg, 5°C, 1h). Subsequently, the pellet was resuspended with aqueous medium, depending on the experiment.

5.2.6. Determination of Encapsulation Efficiency

Uncoated or polymer-coated liposomes (2ml) were ultracentrifuged (150,000xg, 5°C, 1h), the supernatants separated and the EE determined from the γ-counts in the pellet. The EE was expressed as the ratio of the amount of activity found in the pellet to the initial activity of ^{125}IBP1-16 added during preparation of the liposomes.

5.2.7. Determination of Polymer Interaction with Liposomes

Analysis of OPP/CHP coating efficiency of liposomes followed a procedure that was reported previously for carboxymethylchitin (*Dong and Rogers, 1991*). Briefly, 1 ml of the OPP/CHP-coated liposomes were ultracentrifuged and the pellet was diluted to 10 ml with 1:1 v/v isopropanol:acetate buffer solution. After 15 min, absorbances were recorded at 400 nm and the OPP/CHP concentrations were determined from a calibration curve. Absorbances were corrected for the presence of lipids by similarly treating uncoated liposomes. Coating efficiency (CE) was calculated as a ratio of the weight of OPP/CHP (mg) in coated liposomes to the weight of total lipid (mg) in liposomes.

5.2.8. Release Studies of ^{125}I-BP1-16 from Liposomes

Uncoated and OPP/CHP-coated FATMLVs (3 ml) were prepared as before except the pellet was resuspended to 25 ml in simulated gastric (SG) or simulated intestinal (SI) fluids at 37°C. The liposomes were incubated at 37°C (Dubnoff Metabolic Shaker), 250 μl samples were removed at various time intervals up to 12 h, centrifuged (14,000 rpm, 5°C, 5 min) and 100 μl of the supernatant were analyzed for released ^{125}I-BP1-16 (γ-counts). The fraction of BP1-16 released from liposomes, under sink conditions, was determined from,

$$\% \text{ Released} = (C-C_o)/(C_t-C_o) \times 100$$

where C_o is the initial drug concentration in the supernatant before incubation, C_t is the total drug concentration in the liposomes, and C is the concentration in the supernatant at time, t (*Dong and Rogers, 1991*). Statistical comparisons between the fraction of ^{125}I-BP1-16 released from uncoated, OPP and CHP-coated liposomes were made using one-way ANOVA ($\alpha = 0.05$).

5.3. Results and Discussion

The derivatization of pullulan yielded a degree of substitution of either palmitoyl or cholesterol chains proportional to the amounts of palmitoyl chloride or cholesteryl chloroformate added during the reaction. However, the higher the substitution, the less water-soluble was the product. A degree of substitution of 2 palmitoyl chains or 1 cholesterol molecule(s) per pullulan monomer was found to have a satisfactory solubility. The ^{1}H-NMR spectrum of OPP and CHP yielded signals between 0.6 ppm and 2.2 ppm, corresponding to the methyl and methylene protons of the palmitoyl chains and cholesterol molecule, which were absent in the ^{1}H-NMR of pullulan.

5.3.1. Encapsulation Efficiency of BP1-16

Our preliminary investigations with the EE of ^{125}I-BP1-16 suggested that higher loadings occured when a negatively-charged phospholipid, such as DMPG, was present in DOPC:CH or DPPC:CH (3:1 mole ratio) liposomes at 5 to 10 mole percent concentration. The EE was pH-dependent, and, a maximum of 77 and 68 percent EE was obtained in liposomes of DOPC:CH:DMPG and DPPC:CH:DMPG (3:1:0.25 mole ratio, in either case) at pH 5.4, respectively (Table 5.1). These levels of encapsulation are consistent for cationic solutes and negatively-charged liposomes (*Arien et al., 1993*). At pH 9.0, when the peptide possessed a net negative charge, the EE decreased to 37 percent (Figure 5.1), indicating that electrostatic binding of the peptide to the liposome surfaces largely contributes to EE. A lower EE in liposomes of DPPC composition suggest that the binding of ^{125}I-BP1-16 is sterically hindered due to the gel state of these bilayers. Coating the liposomes with OPP or CHP did not significantly alter the EE in either liposome system (Table 5.1).

5.3.2. Coating Efficiency of Liposomes by OPP or CHP

Previously, it was reported that liposomes equilibrated with polysaccharides such as dextran, pullulan, mannan, and amylopectin became coated but the polymer easily desorbed on diluting the liposome dispersions. A solution to this problem was to use polysaccharides that were derivatized with hydrocarbon side chains as hydrophobic anchors, which penetrated the surface bilayers of liposomes (*Sunamoto et al., 1983*). The degree of liposome coating by OPP at pH 5.4 is shown in Figure 5.2 where maximum CE was observed at 0.5 percent and is representative of the results obtained by CHP. This is compaprable to 0.6 percent reported by *Khattab et al.* (*1989*) for silicone adsorption on liposome surfaces. Higher OPP coating of DOPC:CH:DMPG liposomes is indicative of the easier inclusion and penetration of fluid state liposomes by the polymer as its concentration is increased.

5.3.3. Comparative Effects of Polymers on Release of ^{125}IBP1-16

All release profiles were characterized by an initial rapid release phase (0-2 h) followed by a slower, 1st-order kinetics of release (2-12 h). Figures 5.3 and 5.4 compare the relative effectiveness of OPP and CHP to reduce the leakage of entrapped ^{125}I-BP1-16 from DOPC:CH:DMPG (3:1:0.25 mole ratio) liposomes in SG fluids (pH 1.2) and SI (pH 7.4) fluids, respectively. There was a significant ($p < 0.05$) reduction in the amount of ^{125}I-BP1-16 released from OPP/CHP-coated DOPC and DPPC compositions at 12 h and it ranged between 12-44 percent (Table 5.2). Coating liposomes with CHP, however, had a more pronounced effect, reducing the leakage by at least 28 percent after 12 h. Thus, in SG fluid at 37°C, the CHP-coated DPPC composition was more effective in retarding the leakage of the entrapped peptide such that 50 percent of the liposome contents still remained at 12 h.

Figures 5.5 and 5.6 compare the relative effectiveness of OPP and CHP to reduce leakage of entrapped peptide from DPPC:CH:DMPG (3:1:0.25 mole ratio) liposomes in SG and SI fluids, respectively. Again, release of the peptide was faster from DOPC composition liposomes. Indeed, nearly all of the entrapped ^{125}I-BP1-16 was released with in 2 h (Figure 5.4), which compares with 80 percent released from DPPC:CH:DMPG composition (Figure 5.6). The effect of OPP on release was more pronounced in SI than in SG fluids after 2 h (Table 5.2). The release profiles were remarkably similar when liposomes were coated with CHP, however, there was a significant ($p < 0.05$) reduction in the fraction of ^{125}I-BP1-16 released at 12 h. The SI fluids represent a more hostile environment for the liposomes but polymer coating, particularly with CHP, permitted the retention of as much as 40 percent of its contents after 12 h. This is encouraging evidence to attempt to exploit polymer-coated liposomes as an oral delivery system for peptide and protein drugs.

Table 5.1 Encapsulation Efficiency (%) of ^{125}I-BP1-16 in Uncoated, OPP, and CHP-coated Liposomes at pH 5.4[a]

Composition	Uncoated	OPP-coated	CHP-coated
DOPC:CH:DMPG[b]	77.2±1.7	75.6±2.2	76.2±1.8
DPPC:CH:DMPG[b]	67.7±2.7	66.8±1.4	67±1.2

[a] calculated as a fraction of the initial ^{125}IBP1-16 (1.8×10^6 dpm/ml)

in 15 mM total lipid concentration,

[b] 3:1:0.25 mole ratio,

means±SEM, n=6.

Table 5.2 **Percent Reduction in the Fraction of ^{125}I-BP1-16 Released as a result of Coating by OPP and CHP in Simulated GI Fluids at 37°C**

Release Medium	Time (h)	Liposome Compositions			
		DOPC:CH:DMPG[a]		DPPC:CH:DMPG[a]	
		OPP	CHP	OPP	CHP
SG Fluid,	2	20	34	23	38
pH 1.2	12	12	33	18	42
SI Fluid,	2	28	44	29	36
pH 7.4	12	11	28	19	33

[a] 3:1:0.25 mole ratio

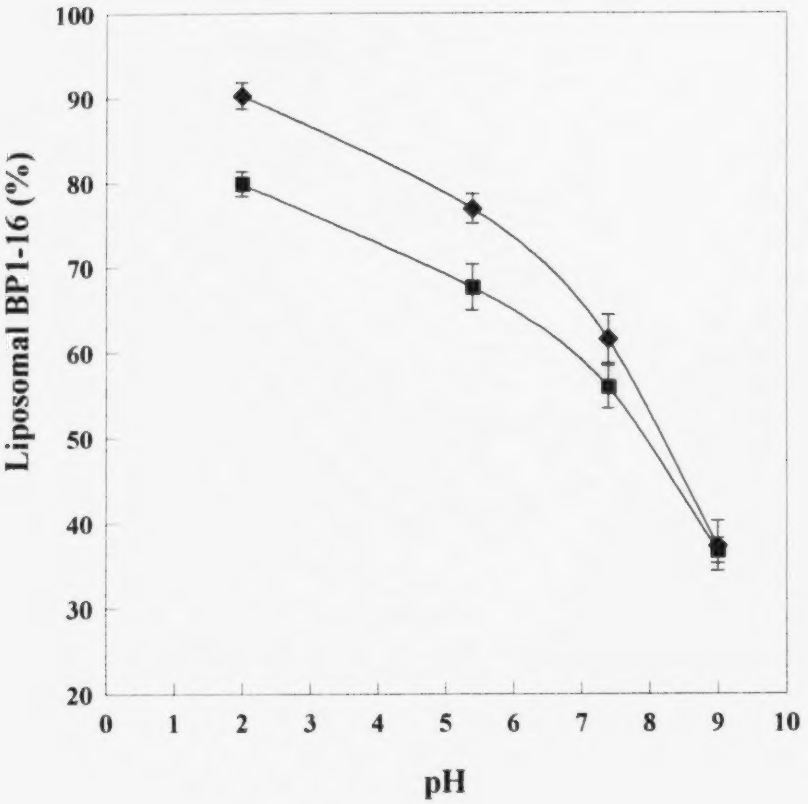

Figure 5.1 Encapsulation efficiency of ^{125}I-BP1-16 in liposomes as a function of pH. ◆, DOPC:CH:DMPG (3:1:0.25 mole ratio). ■, DPPC:CH:DMPG (3:1:0.25 mole ratio); Means±SEM, n=3.

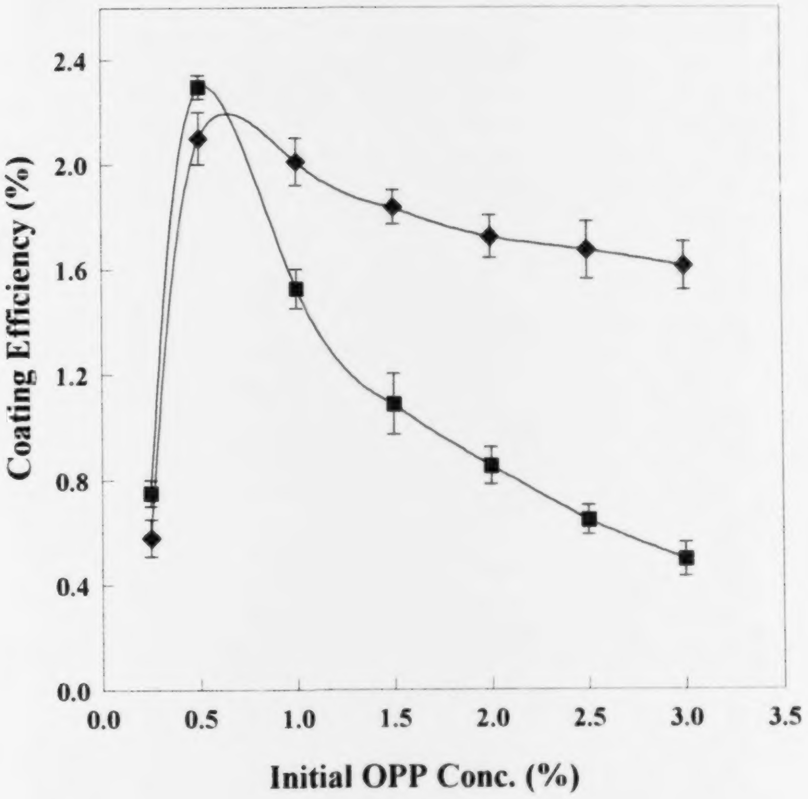

Figure 5.2 Coating efficiency (%) of liposomes by OPP as a function of the initial OPP concentration at pH 5.4. ◆, DOPC:CH:DMPG (3:1:0.25 mole ratio); ■, DPPC:CH:DMPG (3:1:0.25 mole ratio); Means±SEM, n=3.

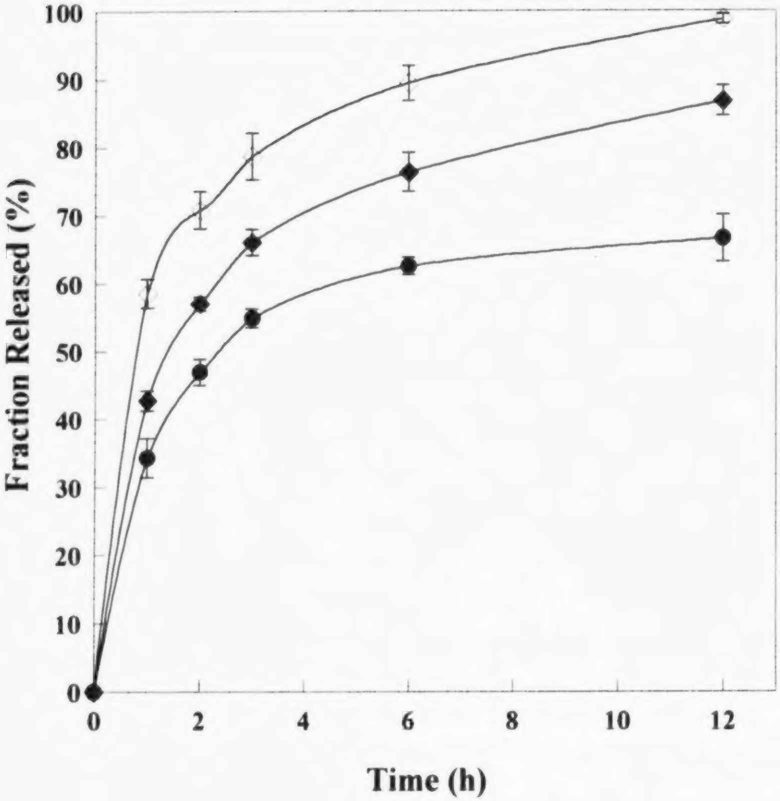

Figure 5.3 Release kinetics of ^{125}I-BP1-16 from uncoated, OPP-coated and CHP-coated DOPC:CH:DMPG (3:1:0.25 mole ratio) liposomes in SG fluid, pH 1.2, 37°C. ◇, Uncoated; ◆, OPP-coated; ●, CHP-coated; Means±SEM, n=3.

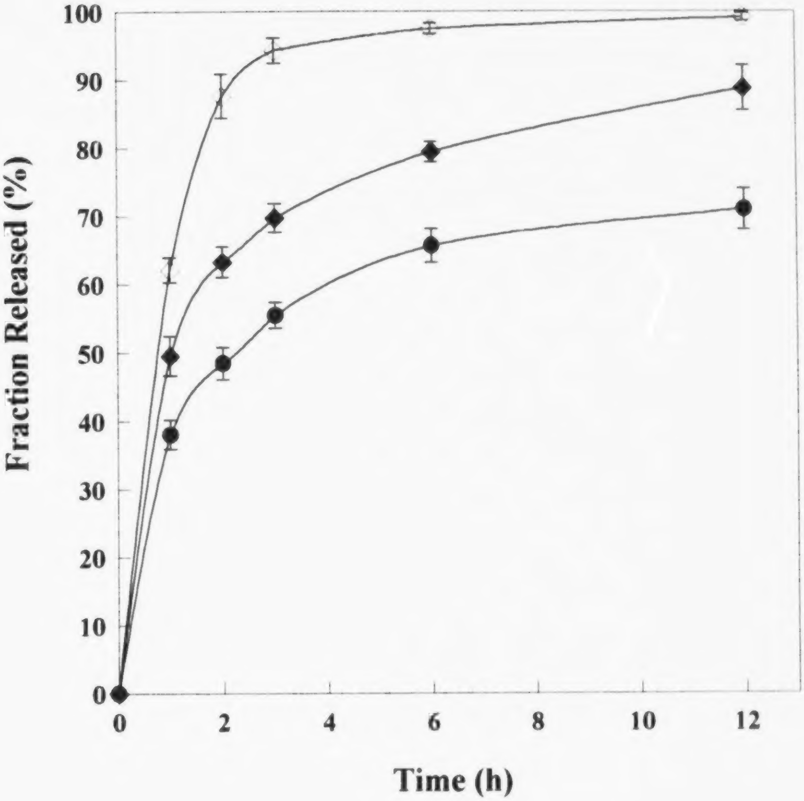

Figure 5.4 Release kinetics of ^{125}I-BP1-16 from uncoated, OPP-coated and CHP-coated DOPC:CH:DMPG (3:1:0.25 mole ratio) liposomes in SI fluid, pH 7.4, 37°C. ◇, Uncoated; ◆, OPP-coated; ●, CHP-coated; Means±SEM, n=3.

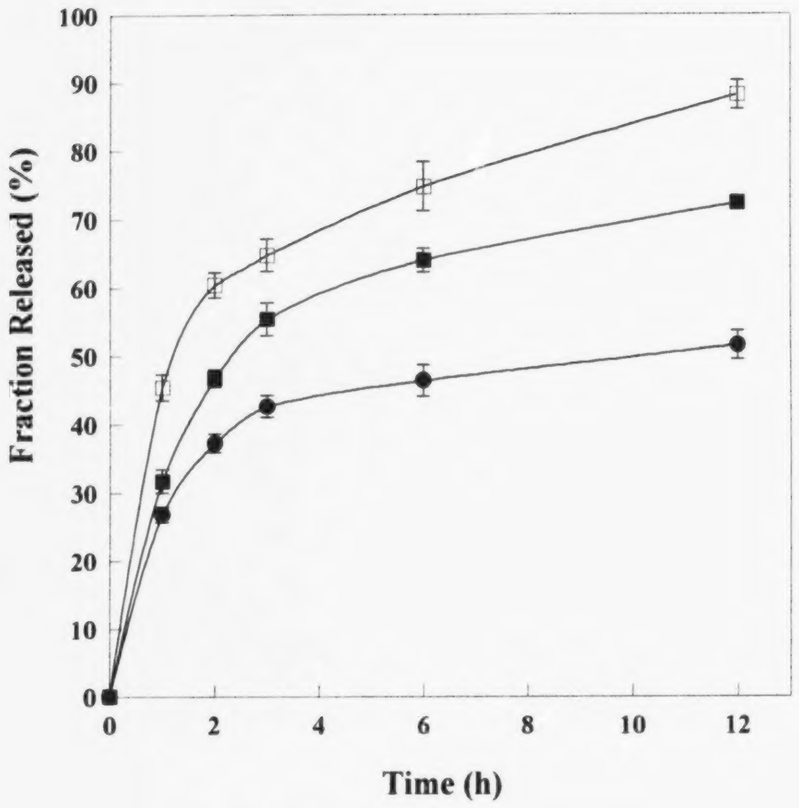

Figure 5.5 Release kinetics of ^{125}I-BP1-16 from uncoated, OPP-coated and CHP-coated DPPC:CH:DMPG (3:1:0.25 mole ratio) liposomes in SG fluid, pH 1.2, 37°C. □, Uncoated; ■, OPP-coated; ●, CHP-coated; Means±SEM, n=3.

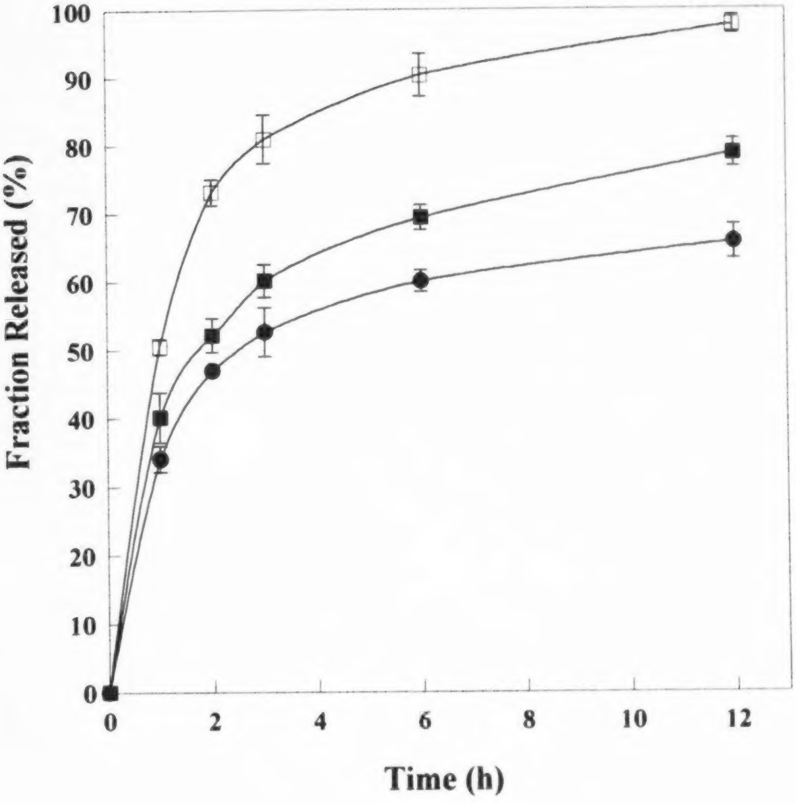

Figure 5.6 Release kinetics of ^{125}I-BP1-16 from uncoated, OPP-coated and CHP-coated DPPC:CH:DMPG (3:1:0.25 mole ratio) liposomes in SI fluid, pH 7.4, 37°C. □, Uncoated; ■, OPP-coated; ●, CHP-coated; Means±SEM, n=3.

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Chapter 6

GENERAL DISCUSSION AND CONCLUSIONS

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6.1. General Discussion

Modern medicine uses many different drug delivery systems (DDS). Besides mechanical devices such as pumps, infusion systems, or topical or implanted patches with controlled leakage rates of drugs, there are also the so-called particulate and soluble drug carriers. The particulate systems can be either local or systemic, *i.e.*, they can be localized at specific sites in the body and release the drug according to a program, such as intelligent hydrogels, or can circulate with body fluids, most often in the general circulation.

Particulate or colloidal DDS include liposomes, polymeric complexes, lipid or polymeric microspheres, micro/nanoparticles, microemulsions, lipoproteins, mixed micelles and other soluble, non-colloidal carriers such as macromolecules, enzymes, antibiotics, and hydrophilic polymers (*Chasin and Langer, 1990*). Such DDS have the advantage of altering the pharmacokinetics and biodistribution of the entrapped drugs along with the possibility of reducing toxicities of some efficacious drugs that are not used because of their high toxicity.

Many studies have been carried out in order to establish the potentials and limitations of liposomes as pharmaceutical dosage forms since they have the ability to carry hydrophilic solutes in their aqueous spaces, within the bilayers (hydrophobic solutes), or even bound to the bilayer surfaces *via* electrostatic interactions. Their biodegradation without any significant toxic by-products, general non-antigenicity coupled with good solubilization power and capacity, relative ease of preparation, and a wide selection of materials and processes allowing tailor-made physicochemical properties make them an attractive drug carrier system (*Lasic, 1993*). Also, with the advent of commercialization of recombinant techniques, the next decade may see a number of liposomal delivery systems for macromolecular drugs. A wide variety of potentially useful macromolecular drugs have been successfully incorporated into liposomes (*Weiner et al., 1989; Nellans, 1991*). Attempts are now being made to improve the liposome retention profile for local sustained delivery applications by modifying the membrane surfaces with agents that carry a determinant for a specific tissue site. Ingredients often selected for these applications include glycolipids, asialoglycoproteins, monoclonal antibodies, and site specific proteins, such as lectins and fibronectin.

Drug-loaded liposomes can be administered by many routes. The most common is parenteral administration, with intravenous administration being used much more than intramuscular, subcutaneous, or intraperitoneal. The oral route of administration encounters problems of liposome instability at low values of pH, and in the presence of digestive enzymes and bile salts, not to mention the phagocytic cells present in the gut. Several investigators have shown poor entrapment of AraC in liposomes but its antitumor activity can be enhanced by encapsulation in liposomes (*Tokunaga et al., 1988*). Another systematic study carried out (*Mayhew et al., 1984*) to observe the effect of cholesterol (CH) on the encapsulation efficiency (EE) of AraC in DPPC liposomes. Ratios of DPPC:CH of 1:0.43 captured 18 % AraC, 1:0.54 captured 20 %, 1:0.67 captured 20.5 %, 1:0.8 captured 22 %, and 1:1 captured 23 percent AraC, respectively. Thus, the amount of AraC captured slightly increased with increase in CH content in DPPC:CH liposomes. In further studies, it was observed (*Mayhew et al., 1984*) that extruded MLVs composed of PG:PC:CH (1:4:5) encapsulated AraC ranging between 5 and 25 percent.

The results showed that as the liposome size decreased, the EE also decreased confirming that most of the AraC was entrapped in aqueous compartments. In another study in which AraC was entrapped in MLVs and SUVs of DSPC:CH (1:1) and sphingomyelin:egg PC:CH (1:4:5) liposomes, EEs between 2 and 15 percent were obtained. The SUVs predominantly showed lower EE than the MLVs. Another group prepared DSPC/DPPC/DMPC:CH:SA (1.33:1:0.12) MLVs (*Patel and Baldeschwieler, 1984*) containing EEs ranging from 16 to 26 percent AraC, which again was no significant improvement over previous reports.

The addition of charged lipids, such as DCP, PA, DMPG, SA often improves the EE of hydrophilic solutes by virtue of increased aqueous compartments due to electrical repulsion of charged bilayers (*Westman et al., 1982*). The presence of negatively-charged polar head groups also increases liposome stability and reduces chances of vesicle aggregation and fusion (*Magin et al., 1990*). As a result, EEs of 28 and 40 percent AraC were obtained in uncoated DMPC:CH:DCP (3:1:2 mole ratio) and DPPC:CH:DCP (3:1:0.5 mole ratio) composition liposomes, respectively (Table 2.1). Furthermore, it was revealed that AraC was mainly electrostatically bound to the more accessible fluid state bilayers, whereas a large portion of AraC was entrapped in the aqueous compartments in gel state bilayers (Figure 2.1). It has been known for some time that the EE of hydrophilic solutes can be improved substantially by applying freeze-thaw (FT) cycles due to repetitive breakage and reformation of lipid membranes, thus increasing drug loading from the aqueous environments (*Mayer et al., 1985*), and these FATMLVs have been reported to be osmotically stable (*Mayer et al., 1985*). Using this approach, liposome compositions entrapped ^{125}I-BP1-16, a small peptide (23 aa residues, pI 7.1), with EE reaching 77 and 68 percent in DOPC:CH:DMPG and DPPC:CH:DMPG compositions (3:1:0.25 mole ratios), respectively. The EE was pH-dependent (Figure 5.1) and decreased at higher pH values due to a net negative charge on the peptide, and hence, being electrostatically repelled by anionic bilayer surfaces. A slightly higher entrapment of ^{125}I-BP1-16 in DOPC:CH:DMPG liposomes most likely involves hydrophobic interaction of the peptide within the bilayers (*i.e.* transmembrane orientation). Also, the fluid state bilayers (DOPC) can accommodate various peptide conformations with relative ease than the gel state (DPPC) liposome composition.

Structural stabilization of liposomes can be accomplished to varying degrees not only by the selected components of the bilayers but also by surface macromolecules, such as polysaccharides (*Sunamoto and Iwamoto, 1985*). The idea has evolved from a recognition of the structure of plant cell membranes, which are coated with naturally-occuring polysaccharides, such as pullulan, dextran, amylopectin, mannan and levan. Interactions between derivatized polysaccharides and liposomal membranes have been investigated (*Iwamoto and Sunamoto, 1982*), and it has been shown that polysaccharides have the ability to adhere to bilayer surfaces, mainly by hydrophobic interactions. Thus, chemically conjugated palmitoyl or cholesterol pullulans become intercalated with lipid bilayer membranes through noncovalent interactions arising from their hydrophobic anchors. The presence of an anchor group rather than the molecular weight of the polysaccharide appeared to be more important in coating liposome surfaces. Such polysaccharide-coated liposomes showed improved stabilities against enzymatic lysis and reduced permeabilities of entrapped water-soluble solutes, such as carboxyfluorescein (*Sunamoto et al., 1984*).

The coating efficiency (CE) by OPP and CHP was maximum at 0.5 percent initial polymer concentration and decreased at higher concentrations, more so for the gel state liposomes. The OPP and CHP-bilayer interactions were confirmed by DSC (Figures 3.5 and 4.8). Coating DPPC:CH:DCP (3:1:0.5) liposomes with CHP decreased the $\delta T_{1/2}$ from 26 to 15.6 (Table 4.2) confirming the fact that the polymer provided structural stabilization to the bilayers. Similar observations were made by others who reported maximum polymer-lipid bilayer interaction at 0.6 percent (*Khattab et al., 1989*) and also 0.1-1.2 percent concentration (*Sato, 1990*). The degree of penetration of the hydrophobic anchor is largely influenced by membrane fluidity. Thus, the fluid liquid-crystalline state DMPC or DOPC liposome compositions easily accommodated the palmitoyl or cholesterol anchors in their membrane domains compared to gel-state liposomes, and hence, a higher CE was obtained. Furthermore, another CE maximum observed at 2.5 percent OPP/CHP concentration (Figures 2.2 and 4.1) indicated that additional polymer association with the fluid state liposomes may be due to penetration within the bilayers deeper than the surface layers, or possibly due to adsorption of multiple polymer layers at bilayer surfaces.

Coating DMPC:CH:DCP (3:1:2 mole ratio) and DPPC:CH:DCP (3:1:0.5 mole ratio) composition liposomes with 0.5 percent OPP decreased AraC EEs to 15 and 27 percent, while coating with CHP decreased them to 24 and 36.5 percent, respectively. The decrease in EE by OPP-coating is likely due to its restricting effect on the polar head groups of bilayers affecting the binding sites that could have been available for AraC molecules. In comparison, CHP which possesses a larger hydrophobic anchor with a spacer group, had a lesser restricting effect even though a higher ordering within the up to 2 h, followed by first-order kinetics of release extending up to 12 h. Mechanistically, it is proposed that the initial rapid release phase is due to the dissociation of the outermost-bilayer bound solute molecules (AraC or ^{125}I-BP1-16) (k_1, Figure 6.1), and their release into the external medium. Consequently, the molecules from the inner bilayers of MLVs start dissociating and diffusing towards the surface bilayers at a rate, k_2 (Figure 6.1), thereby re-establishing an equilibrium within the surface bilayers. Until such time when the rate of transfer $k_1 > k_2$, a rapid release phase is observed (lasting for approximately 2 h). However, when $k_1 = k_2$, the rate of loss of the solute entrapped within the aqueous compartments follows a first-order kinetics of release process.

When the liposomes are coated by polymers, such as OPP or CHP, the diffusivity of bilayers is altered which can be determined by a rate constant, k_2' (Figure 6.1). The presence of a polymer layer causes displacement of part of the surface-bound solute molecules (which explains the lower EE of polymer-coated liposomes). In addition, the bilayer diffusivity (k_2') to free/bound solutes molecules is decreased, hence, the polymer-coated liposomes have a lower fraction of solute released and also lower 1st-order release rate constants.

The SI fluids represent the most hostile environment for the liposomes but coating them with OPP decreased the first-order release rate constant (k_1) for AraC by 20 and 60 percent in SG fluids, and by 150 and 60 percent in SI fluids for the DMPC:CH:DCP and DPPC:CH:DCP compositions, respectively (Table 3.2). Similarly, coating the liposomes with CHP decreased k_1 by 100 percent in SG fluids for both compositions, and by 30 and 70 percent in SI fluids for the DMPC and DPPC compositions, respectively

(Table 4.3). The reduction in k_1 from polymer-coated bilayers was associated with the condensing effect of polymer chains on outer bilayer surfaces which was confirmed by DSC studies (Figures 3.5 and 4.8).

In case of ^{125}I-BP1-16, however, coating the liposomes with OPP reduced the fraction of peptide released by about 12 and 18 percent in SG and SI fluids for DOPC:CH:DMPG and DPPC:CH:DMPG liposomes, respectively. Similarly, coating the liposomes with CHP reduced the fraction released by 33 and 42 percent in SG fluids, and by 28 and 33 percent in SI fluids at 37°C.

Based on this work and other known studies, some generalizations can be made with respect to the formulation of liposomes as a DDS for oral administration. Factors that contribute to decreased leakage of entrapped solutes include *membrane fluidity* as influenced by cholesterol content, the presence of *charged bilayers* to induce solute binding, the presence of *polymer-coat(s)* that provides structural stabilization to the membranes, and the *gel state* rather than the liquid-crystalline state bilayers.

6.2. Conclusions

1. The EE of charged solutes is influenced by the T_m of the constituent phospholipids, the presence of cholesterol, and the presence of charged adducts that provide binding sites for solute binding. Higher EE in gel-state than fluid-state liposomes were due to their larger aqueous compartments.
2. Derivatized polysaccharides are retained on the liposome surfaces as a monolayer at 0.5 percent initial polymer concentration. At 2.5 percent concentration, another maximum is postulated as being due to deeper penetration of the outer liposome bilayers and/or multilayered polymer adsoption in case of fluid-state liposomes.

3. The CHP polymer had a less solute-displacing effect from liposome surface bilayers than OPP because of the presence of a spacer group (AECM moiety) that anchored the pullulan chains.
4. The gel-state, than the fluid-state liposomes, possessed greater stability in the presence of cholate ions at pH 7.4 due to their inability to penetrate the more rigid negatively-charged gel-state bilayers.
5. Coating the liposomes with OPP or CHP significantly reduced the initial rapid release phase which was due to the dissociation of outer-bilayer bound solute molecules, and the subsequent first-order release phase which resulted from the diffusion of entrapped solute through the bilayers. Coating with CHP, than OPP, was more effective in altering bilayer permeability.

6.3. Future Work

Future studies should concentrate on derivatizing other water-soluble polymers, such as mannans, levans and poly(carboxylic acids), with the intention of adding an anchor group or providing ionic interactions between polymer and lipid membranes. At a time when an optimal formulation has been identified, GI transit studies and targeting to absorption sites should be undertaken.

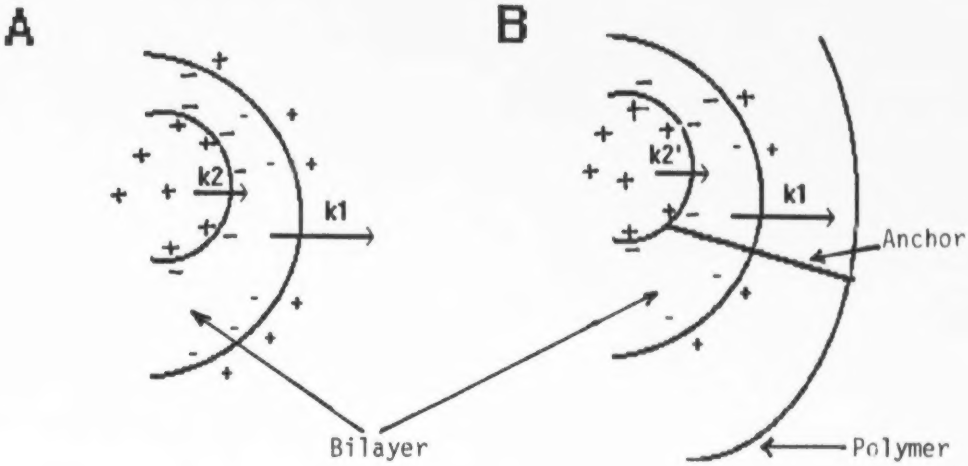
A
B
k2
k1
k2'
k1
Anchor
Bilayer
Polymer

Figure 6.1. Proposed schematic representation of: **A**. Uncoated lipid bilayer, and **B**. Polymer-coated lipid bilayer *via* a hydrophobic anchor. The negative and positive charges denote the presence of anionic bilayers and surface-bound solute molecules, respectively.

6.4. References

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